EFFECT OF COSOLVENTS ON THE STRUCTURE, FUNCTION AND STABILITY OF SELECTED ENZYMES

A THESIS SUBMITTED TO THE UNIVERSITY OF MYSORE,

MYSORE, FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN

BIOCHEMISTRY

BY

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OCTOBER, 2008

Dedicated to My Beloved Guru

DECLARATION

I hereby declare that the thesis entitled **"Effect of cosolvents on the structure, function and stability of selected enzymes"** which is submitted herewith for the degree of **Doctor of Philosophy** in **Biochemistry** of the **University of Mysore, Mysore** is the result of research work done by me in the Department of Protein Chemistry and Technology, Central Food Technological Research Institute, Mysore, India under the guidance of **Dr. V. Prakash,** Director, Central Food Technological Research Institute, Mysore during the period 2004-2007.

I further declare that the results of this work have not been previously submitted for any degree or fellowship.

Date: 6th October, 2008 **Mr. Gangadhara**

Place: Mysore

CERTIFICATE

I hereby certify that the thesis entitled **"Effect of cosolvents on the structure, function and stability of selected enzymes"** submitted by **Mr. Gangadhara**, for the degree of **Doctor of Philosophy** in **Biochemistry** to the **University of Mysore, Mysore** is the result of research work carried out by him in the Department of Protein Chemistry and Technology, Central Food Technological Research Institute, Mysore, India under my guidance and supervision during the period June 2004-June 2007.

I further declare that the results of the work have not been submitted either partially or fully to any other degree or fellowship.

Date: 06.10.2008

Place: Mysore

V. Prakash

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ACKNOWLEDGEMENTS

With a deep sense of gratitude, I express my sincere and heartful thanks to my guide and mentor Padma Shri Dr. V. Prakash, Director, Central Food Technological Research Institute, Mysore, India for suggesting the research problem, valuable guidance, constant encouragement and constructive criticism during the course of this investigation and preparation of thesis. I will cherish the memories of my association with such an elegant personality.

It gives me immense pleasure to express my heartful thanks to him as Director, Central Food Technological Research Institute, Mysore, for providing all the facilities to enable me to carry out my research work in the institute.

I am grateful to Dr. A. G. Appu Rao, Head, Department of Protein Chemistry and Technology at CFTRI and fellow colleagues for providing me with tremendous encouragement and lab facilities during my stay in the department. I thank all the staff of library and central instrumentation facility and service at CFTRI for the help they have extended to assist during the research period.

I would like to specially thank Dr. Ramesh Kumar, Dr. (Mrs.) Purnima Kaul and Dr. (Mrs.) Radha who despite their busy schedule, always went out of their way to help me. Discussions with my lab mates Devaraj, Jaykant, Gunasekhar and Anuradha have always helped in critically evaluating my work. I am thankful to them for their support and co-operation.

A number of colleagues and friends have always extended their helping hand at various stages during the course of this work. I would here like to take this opportunity to express my sincere gratitude to all those people.

To build a career in science it is imperative to have assurance and sacrifice from the family. I am grateful to my parents and brothers for the love and care extended to me during my career.

The Senior Research Fellowship from Council of Scientific and Industrial Research, New Delhi is gratefully acknowledged.

Date: 6th October, 2008 **Mr. Gangadhara**

Place: Mysore

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LIST OF ABBREVIATIONS

A	absorbance
Å	angstrom
ANS	8-anilino-naphthalene sulfonic acid
Asp	aspartic acid
BSA	bovine serum albumin
[C]	concentrations of protein (mg/ml)
°C	degree Celsius
$CaCl_2$	calcium chloride
CD	circular dichroism
cm	centimeter
DNA	deoxyribonucleic acid
DNS	3,5-dinitrosalicylic acid
DEAE	diethylaminoethyl
EDTA	ethylene diamine tetra acetic acid
Fig	figure
F_u	fraction unfolded
g	gram
Glu	glutamic acid
GuHCl	guanidine hydrochloride
h	hour
HCl	hydrochloric acid
His	histidine
K_a	association constant (M^{-1})
°K	degree Kelvin

KCl	potassium chloride
kDa	kilodalton
kCal	kilocalories
k_{cat}	catalytic constant
Kg	kilogram
K_i	inhibition constant
K_m	Michaelis constant
l	litre
Li_2SO_4	lithium sulfate
M	molar concentration
mA	milli ampere
mg	milligram
min	minute
ml	milliliter
mm	millimeter
mmol	millimoles
mol	mole
MRW	mean residue weight
MW	molecular weight
N	normality
NaCl	sodium chloride
NaOH	sodium hydroxide
ng	nanogram
nm	nanometer
P	pressure

PPL	porcine pancreatic lipase
PAGE	polyacrylamide gel electrophoresis
pI	isoelectric point
R	universal gas constant
RBL	rice bran lipase
RNA	ribonucleic acid
rpm	revolutions per minute
SD	standard deviation
SDS	sodium dodecyl sulfate
sec	second
SeO_2	selenium dioxide
SH	thiol groups
Ser	serine
SS	disulfide
T	absolute temperature
TCA	trichloro acetic acid
TEMED	*N,N,N',N'*,-tetramethyl ethylene diamine
T_m	thermal mid point transition temperature
Tris	tris (hydroxymethyl) amino methane
Trp	tryptophan
Tyr	tyrosine
UV	ultraviolet
v/v	volume by volume
$\bar{v}$	partial specific volume of protein (ml/g) extrapolated to zero protein concentration

$\bar{v}_{app}$	apparent partial specific volume (ml/g) at a single protein concentration
V_e	elution volume
V_{max}	maximal velocity
V_t	total volume
V_0	void volume
w/v	weight by volume
w/w	weight by weight
$ZnCl_2$	zinc chloride
μ	chemical potential
μg	microgram
μl	microlitre
μmol	micromole
%	percentage
λ_{max}	emission maximum (wavelength)
$E^{1\%}_{1cm\ \lambda max}$	absorption coefficient of 1% solution in 1 cm path length cell at its absorption maximum
ε	molar extinction coefficient ($M^{-1}cm^{-1}$)
ρ_0	density of solvent buffer
ρ_P	density of protein solution
ξ_3	preferential interaction parameter of protein
ϕ_2^0	isomolal partial specific volume of protein (ml/g) extrapolated to zero protein concentration
$\phi`_2^0$	isopotential partial specific volume of protein (ml/g) extrapolated to zero protein concentration
ΔV	volume change upon transfer of one mole of protein from one solvent system to another (ml/mol)

$\Delta G°$	free energy change of the reaction when all of its reactants and products are in their standard states
$(\delta g_3/\delta g_2)$	preferential interaction parameter on g/g basis
$(\delta m_3/\delta m_2)$	preferential interaction parameter on mol/mol basis

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INTRODUCTION

INTRODUCTION

Enzymes are Nature's catalysts. Millions of years of evolution have optimized enzymes to catalyze a myriad of reactions essential for life. As a consequence, most of these reactions occur at conditions favorable to life: aqueous solvent, mild temperature, atmospheric pressure, afnd near neutral pH. Due to the delicate balance of stabilizing and destabilizing interactions, enzymes are marginally stable. Stability of enzyme activity *in vitro* reflects the molecular integrity of the constituent polypeptide chains. Understanding the various factors responsible for the stability and activity of enzymes in solutions is an academic challenge; with an enormous implications for the pharmaceutical and the biotechnology industry. Considerable time in nearly all fields of biochemical sciences is devoted to improve the enzyme stability which is the result of a balance between the intramolecular interactions of protein functional groups and their interaction with solvent environment.

Identification of intrinsic and extrinsic factors which contribute to the stabilization of enzymes can provide valuable information for better stability. Therefore, enzyme stabilization under different conditions is an important area of research. The different methods employed for enzyme stabilization are immobilization, chemical modification, protein engineering and inclusion of various additives as enzyme stabilizers (Russell and Klibanov, 1988; Sears *et al.,* 1994; Almarsson and Klibanov, 1996; Kuchner and Arnold, 1997). Use of stabilizing additives is a customary practice in enzyme technology and shelf life of enzyme products are very much relying upon such additives. These molecules stabilize enzymes, not by interacting with them directly but by altering the solvent properties of the surrounding water. A variety of solvents have been shown to affect the stability. Among the solutes employed for this purpose are sugars, polyols, salts and amino acids.

A few general schemes for the mechanisms of action of these cosolvents have been proposed (Timasheff, 1998; Bolen and Baskakov, 2001; Bolen, 2004). The cosolvent-protein interaction is a surface phenomenon where more water is structured around the protein molecule, a phenomenon known as preferential hydration leading

to stabilization of protein. The driving force is the perturbation by the protein of the chemical potential of the cosolvent. It is shown that the measured change of the amount of water in contact with protein during the course of the reaction modulated by a cosolvent is a change in preferential hydration that is strictly a measure of the cosolvent chemical potential perturbation by the protein in the ternary water-protein-cosolvent system (Timasheff, 2002b). Cosolvents can affect the aqueous environments in numerous ways; for example alter viscosity, density, dielectric constant, refractive index, pH and osmotic pressure.

Cosolvent systems can be divided into four categories depending on whether their effect on protein transition is neutral, favorable, unfavorable or combined (McClements, 2002). Neutral cosolvents are rather rare in real-life situations. This theoretically would occur if cosolvent and solvent molecules had the same size so that steric exclusion and differential interaction were negligible or if steric exclusion and differential interactions compensate each other. Stabilizing cosolvents oppose a protein transition. The origin of the mechanism is based on the fact that the cosolvent system acts as a stabilizer because the change in transfer free energy for the respective transition is positive. Some of the anions and cations such as lithium, sodium and sulfate and many simple sugars (for example, sucrose and trehalose) and polyols (for example, glycerol, xylitol and sorbitol) fall into the category of stabilizing cosolvents. Simple sugars are widely believed to stabilize proteins through steric exclusion mechanisms. Destabilizing cosolvents favor a protein transition. These cosolvents are believed to preferentially bind to the protein surface, thus favoring the unfolded state which exposes more surface area. Commonly used protein denaturation agents such as urea and guanidine hydrochloride fall into this category. Combined cosolvent systems may stabilize a particular state of a protein under some conditions but destabilize in other conditions for example, temperature and cosolvent concentration. One of the main objectives of the present thesis is to understand structure-function and kinetics of enzymes from different families in presence of selected cosolvents and how these insight help in understanding stability of these selected enzymes in presence of similar cosolvents.

From the above angle, to understand the mechanism of cosolvents mediated stability of enzymes in detail, a detailed investigation is undertaken on the stability of

a set of three well characterized enzymes belongs to hydrolases (invertase, nuclease P1 and lipase) at varying cosolvent concentrations, temperature and pH values and interaction studies with rice bran lipase using metal ions and selected biological ligands for inhibitory effects. The combined results from structural stability, kinetics and preferential interaction would give us some insight to understand the mechanism of stabilization of these enzymes in presence of cosolvents.

1.1. Structural stability of enzymes in cosolvents

Proteins play a fundamental role throughout biological process. From catalysis to enzymatic reactions, and from atom-binding to molecule transport, they perform a wide variety of functions inside and outside the cell (Dobson, 2003). The unique native structure is for most proteins a basic requirement for proper functioning. Understanding how a protein's environment in solution affects its stability and thus its activity is important for many reasons, including an understanding of the basic thermodynamics of the process of folding and for the practical reasons that production, processing and utilization of proteins in nature and in biotechnological processes occur under solution conditions far removed from dilute aqueous buffers.

The stability of an enzyme is affected by many factors such as, temperature, pH, oxidative stress, solvent, binding of metal ions or co-factors, and the presence of surfactants (Eijsink *et al.,* 2005). The maintenance of protein/enzyme structure as well as its function is of primary importance from both its understanding and mechanism point of view.

Protein stability plays an extremely important role not only in its biological function but also in protein engineering including protein design, refolding, storage and transfer (Chi *et al.,* 2003). When compared to the physiological conditions, isolated proteins are usually subjected to severe environmental conditions which might lead to inactivation, denaturation and aggregation. Particularly, irreversible protein aggregation is a common feature in protein engineering, and much effort has been devoted to investigating the mechanism and driving force of protein aggregation (Chi *et al.,* 2003) and refolding proteins from aggregates (Meersman and Heremans, 2003) in recent years.

The term stability refers to a protein's resistance to adverse influences such as heat or denaturants, that is, to the persistence of its molecular integrity or biological function in the face of high temperatures or other deleterious influences. A perfectly folded, fully functional protein can lose its biological activity *in vitro* by unfolding of its tertiary structure to a disordered polypeptide in which key residues are no longer aligned closely enough for continued participation in functional or structure-stabilizing interactions. Such unfolding is termed denaturation. It is usually cooperative and may be reversible if the denaturing influence is removed, since the polypeptide chain has not undergone any chemical changes (Mozhaev and Martinek, 1982).

Proteins are also subject to chemical changes leading to an irreversible loss of activity or inactivation, particularly following unfolding. Unfolding may result in the loss of a functionally essential cofactor from a holoprotein such that biological activity will not be regained, even if the unfolding can be reversed to yield the corresponding apoprotein. Unfolded polypeptide chains may aggregate to form an inactive, insoluble mass while an individual chain 'attempting' to refold may enter an incorrect, kinetically trapped conformation from which it cannot emerge. Sometimes these events can be prevented. There exist a number of so-called chaperone proteins which assist the folding of newly synthesized proteins *in vivo*. Chaperones can also act *in vitro* to prevent aggregation and assist folding. At least one such protein can reactivate an aggregated enzyme. Peptide bonds and amino-acid side chains are chemically reactive and can participate in deleterious reactions, particularly at high temperatures of storage conditions (Martin *et al.,* 1993a & b).

These different molecular phenomena give rise to two distinct definitions of *in vitro* protein stability. These are thermodynamic (or conformational) stability and long-term (or kinetic) stability. Thermodynamic stability concerns the resistance of the folded protein conformation to denaturation while long-term stability measures the resistance to irreversible inactivation (i.e., persistence of biological activity) (Mozhaev, 1993).

Thermodynamic stability is mainly measured by techniques such as, UV spectrophotometer, fluorescence and circular dichroism spectroscopy. These methods

are sensitive to changes in protein conformation and thus monitor unfolding of the protein. The unfolding transition is often cooperative, with dramatic changes occurring over a very narrow range of temperature or within a narrow concentration of the denaturant. Empirical stability parameters such as the transition midpoint of thermal unfolding can easily be estimated from experimental data (Schmid, 1989; Baldwin and Eisenberg, 1987). Long-term stability measurement involves assay of biological function (e.g., catalytic activity of an enzyme) by appropriate methods. One places the protein at an elevated temperature and removes samples at intervals, cooling them on ice and then assaying activity under optimal conditions. One may then plot residual activity against time (Malcolm *et al.,* 1990). In summary, there are many indices of protein stability. One should choose whichever is most suitable for one's purposes and avoid inappropriate comparisons.

Many strategies have been employed to increase enzyme stability under different conditions, including protein engineering (Sears *et al.,* 1994; Kuchner and Arnold, 1997) molecular imprinting (Russell and Klibanov, 1988) organic buffering (Blackwood *et al.,* 1994) addition of denaturing cosolvents to the organic solvent to increase the flexibility of enzymes (Almarsson and Klibanov, 1996) and incorporation of carbohydrates, polymers and inorganic salts into the reaction medium (Dabulis and Klibanov, 1993). A variety of attempts are also done to elucidate the nature of stabilization of proteins (Brock, 1985). Structural factors such as salt bridges, hydrogen bonding, ligand binding, hydrophobic interactions, disulfide bonds and amino acid composition also play a major role in the stabilization of enzymes (Kristjansson and Kinsella, 1991). Development of techniques to protect proteins, especially enzymes, against denaturing stresses is always one of the major topics of protein science.

A variety of compounds have been shown to affect the stability and folding of proteins in aqueous solutions (Timasheff, 1998; 2002b). Among the cosolvents utilised for this purpose are sugars, polyols, salts and amino acids. The properties of cosolvents are becoming increasingly useful in molecular biology, agriculture and biotechnology (Cushman, 2001; Yancey, 2001). For example, Welch and Brown, (1996) have suggested that stabilizing osmolytes, which they call 'chemical chaperones', might rescue misfolded proteins in human diseases. Recently, they found

that addition of various mammalian osmolytes and trimethylamine n-oxide can indeed restore function of one form of cystic fibrosis mutant protein. Crop plants are also being engineered to accumulate a variety of so-called compatible solutes for stress conditions (Cushman, 2001).

The origin of the mechanism of stability in presence of cosolvents is based on the fact that the cosolvent system acts as a stabilizer because the change in transfer free energy of the respective transition is positive. The protective mechanism of cosolvents has traditionally been attributed to 'preferential hydration' of the protein (Gekko and Timasheff, 1981a & b; Timasheff, 2002a) which suggested that the exclusion of the cosolvent molecules from the protein surface led to a minimization of the protein surface without changing its conformation.

Sugars and polyols, lead to the increase in the thermal stability of proteins and the increase in the surface tension of solvent water in their presence is considered as a contributory force for their preferential hydration effect and hence protein stability (Lee and Timasheff, 1981; Kaushik and Bhat, 1998; 2003). From the results of hydrogen/deuterium (H/D) exchange studies, Bolen and his coauthors (Bolen, 2001; Bolen and Baskakov, 2001) further suggested a mechanism of 'solvophobic thermodynamic force' which indicated that the unfavorable interaction between the osmolytes and the peptide backbone raised the free energy of the denatured state and as a result, protected the protein by shifting the equilibrium in favor of the native state.

In the case of salts and amino acids which have also been found to lead to an increase in the surface tension of water, the net stabilizing effect is governed by a fine balance between the increase in the energy required for cavity formation in such a medium on protein denaturation, and their ability to bind proteins to varying extents in some cases depending on the pH of the medium (Arakawa and Timasheff, 1983; Lin and Timasheff, 1996). However, the surface tension mechanism does not seem to be applicable in some cases, wherein an increase in the surface tension of aqueous solutions by the addition of solutes has been observed while there is a decrease in the stability of proteins in their presence and vice versa (Bolen, 2004). However,

extensive pH dependence studies on the thermal stability of a number of proteins in organic osmolytes have suggested that the stabilizing effect should also depend on the nature of the amino side chains in proteins and that the structure of aqueous solution plays a central role in the stabilization process (Kaushik and Bhat, 1998; 2003).

1.2. Enzymes and denaturants

Understanding the structure-function relationships of an enzyme under different solvent conditions is fundamentally important for both theoretical and application aspects. Such studies may provide insight into the molecular basis of the stability of the enzyme, which in turn can be used to design protocols and/or a protein with special properties for biotechnological applications. The stability of a native protein is a function of external variables such as ionic strength and solvent composition as they disrupt the different kinds of bonds that are responsible for the intrinsic stability. Therefore, a quantitative analysis of the role of such variables in the formation of the structure of the protein is a prerequisite in describing the forces that are responsible for the conformational stability. A simple method for such studies involves the monitoring of conformational changes due to perturbation of a protein molecule by various agents such as GuHCl and urea (Privalov, 1979).

1.2.1. Interaction with denaturants

Denaturants such as GuHCl and urea are powerful tools in ascertaining the kinetics and energetics of the events that lead to formation of native structures. When proteins are exposed to increasing denaturant concentrations, it is generally observed that their structure does not suffer drastic changes until a certain concentration is reached (Jiang and Tsou, 1994; Tsou, 1995). These findings illustrate that protein denaturation is a cooperative phenomenon that involves the transition from the native to the unfolded state in a single step, i.e., the two state model, where only folded and unfolded states of the protein exist in rapid equilibrium with no observable intermediates or involving multiple steps as the case many be.

However, the development of a wide range of sensitive techniques has led to the detection as well as characterization of intermediates in the folding of several proteins, which were previously believed to obey the two-state model. It has been also

shown in the case of many proteins that folding involves a discrete pathway with the formation of intermediate states between native and denatured states (Kim and Baldwin, 1990; Ptitsyn, 1995). Such intermediates are found to possess special characteristics, which is evident from the spectroscopic and hydrodynamic measurements. Identification and characterization of intermediate states populated during the folding process of many proteins have received considerable attention, and much effort is being spent on this objective.

The results of kinetic experiments with some of the proteins indicate the rapid formation of intermediates with properties consistent with those of the molten globule state, supporting the idea that the molten globule state may be an obligatory intermediate on the folding pathway (Ptitsyn, 1987). The molten globule (MG) is a well known example of a partially folded equilibrium state (Ptitsyn *et al.,* 1990; Mizuguchi *et al.,* 2000). MG states have been proposed to be involved in a number of physiological processes, such as protein recognition by chaperones, release of protein ligands and protein translocation across bio-membranes (Ptitsyn, 1995).

The role of the molten globule state as a functional entity in protein folding is hypothesized, and further evidence is also shown that such state is involved in several biological or pathological processes such as membrane insertion, transmembrane trafficking and chaperone-assisted refolding that require the protein to become partially unfolded (Hartl *et al.,* 1994). Further, point mutation may convert the native protein into the molten globule-like state, and some of these mutations are correlated with the genetic predisposition for human diseases (Matthews *et al.,* 2000). Thus, exploring the structure and dynamics of the molten globule state of a protein is necessary to understand the mechanism of the protein folding.

1.3. Interaction of other ligands with enzymes

Since many enzymes undergo structural transition in presence of metal ions and plant secondary metabolites such as azadirachtin, the studies on the inhibition of RBL in absence and presence of metal ions and plant secondary metabolites assumes significance.

1.3.1. Interaction with metal ions

The effect of Lithium and selenium on RBL activity and its mechanism is not known till now. In order to gain insight into the effects and mechanism of metal ions on RBL activity and the location of their binding sites, we have studied the kinetic effects of monovalent and tetravalent cations on RBL by using lithium and selenium as models of inhibition. This is thought to be the first attempt to explain the role of monovalent and tetravalent cations on RBL activity based on the structure and function of the enzyme. One of the subjects of this study is focused to partly understand the structure-function relationship of rice bran lipase in presence of lithium and selenium ions. Metal ions serve a variety of functions in proteins, the most important of which are to enhance the structural stability of the protein in the conformation required for biological function and/or to take part in the catalytic processes of enzymes. Metal ions can activate chemical bonds and make them more amenable to reaction. They can take part in trigger and control mechanism by specifically altering or stabilizing a macromolecular conformation on binding. But some of the metal ions are known to inhibit certain enzymes (Villeret *et al.,* 1995).

1.3.2. Interaction with azadirachtin

The neem tree, *Azadirzchta indica*, is a tropical plant that is well known for its pesticidal and medicinal properties (Schmuttener, 1990). Many studies have demonstrated that its seed contains abundant limonoids and simple terpenoids that are responsible for its biological activity (Govindachari *et al.,* 1995; Kumar and Parmar, 1996). Among these limonoids, azadirachtin is considered to be the most important active principle due to its various effects on insects (Dai *et al.,* 2001) and has gained considerable attentions as a potential nontoxic, biodegradable and natural pesticide (Ley, 1994). Furthermore, azadirachtin also has great application in herbal medicine/healthcare products, especially for major skin diseases, anti-malarial, anti-tuberculosis, anti-worms, anti-clotting, blood detoxifier, anti-viral, anti-periodontitic, anti-bacterial, anti-fungal, etc., There have been many studies towards understanding the mode of action of azadirachtin, a tetranortriterpenoid, the principle active ingredient on enzymes (Pravin Kumar *et al.,* 2007; Senthil Nathan *et al.,* 2006). Azadirachtin affect enzymes in many different ways, but their modes of action are relatively unstudied in lipase. The effect of azadirachtin on RBL activity and its

mechanism is not known. To examine the effect and mechanism of azadirachtin on RBL activity, we conducted inhibition study of azadirachtin on RBL. One of the objectives of this study is focused to partly understand the structure-function relationship of RBL in presence of azadirachtin ultimately perhaps has an application in the stabilization of rice bran.

1.4. Invertase

Glycosidases are a broad group of enzymes that catalyze the hydrolysis of glycosidic bonds. They play a central role in a number of biological processes that are of significant interest in biochemistry, medicine and biotechnology. Invertases are found in family GH32 of the sequence-based classification of glycoside hydrolases. This family contains more than 370 members from plant, fungal and bacterial origin.

Invertase, the β-D-fructofuranosidase (EC 3.2.1.26) that cleaves sucrose into fructose and glucose is one of the earliest discovered enzymes. It was isolated in the second half of the 19th century, and its name was coined because the enzyme produces "invert" sugar, which is a 1:1 mixture of dextrorotatory D-glucose and levorotatory D-fructose (Koshland and Stein, 1954). Its long history in scientific literature is attributed to its abundance in yeast which had been recognized as the agent responsible for fermentation. Further, the observation that extracts of yeast was capable of producing alcohol from sucrose attracted both the chemists and the biologists to study the mechanism of the reaction and the nature of the enzymes involved. The majority of research concerning yeast invertases has focused on *Saccharomyces cerevisiae* (Carlson and Botstein, 1982; Carlson *et al.,* 1983). Molecular biology techniques have allowed the rapid advancement of our understanding of many non-*Saccharomyces* types of yeast. One of the yeasts *Candida utilis* uses a variety of inexpensive carbon sources (Boze *et al.,* 1994).

Invertase from *Candida utilis* is a glycoprotein and is composed of two identical subunits with a molecular mass of 150 kDa each. Both subunits contained approximately 60% carbohydrate with an isoelectric point of pH 3.35, UV absorption value of 1.8 at 278 nm (Chavez *et al.,* 1997; Belcarz *et al.,* 2002). From the structural model for family 32 of glycosyl-hydrolase enzymes is based on the beta-structure (Pons *et al.,* 1998). After over a century of investigations and almost 40 years since

the first crystal structure of a protein was solved, no three-dimensional structure of an invertase or of any member of glycosidase family GH32 has been reported. Alberto *et al.,* (2004) reported the first three-dimensional crystal structure of *T. maritime* invertase (Fig. 1). The crystal structure determined at 2 Å resolution reveals two modules, namely a five-bladed β-structure with structural similarities to the β-structure of glycosidase from families GH43 and GH68 connected to a β-sandwich module. Three carboxylates at the bottom of a deep, negatively charged funnel-shaped depression are essential for catalysis and function as nucleophile, general acid, and transition state stabilizer respectively. The structure not only provides a template for all members of family GH32, but it also allows dissection of the exquisite details that distinguish retaining and inverting furanosidases with perfectly superimposable catalytic machinery. From the crystal structure of invertase from *T. maritime*, catalytic active site is positioned at one end of the cavity at the center of the β-structure with a funnel-like opening toward the molecular surface (Alberto *et al.,* 2004).

The breakdown of sucrose is widely used as an energy source by bacteria, fungi and plants. In plants, both glucose and fructose are implicated in the signaling pathways by which sucrose concentration functions as a key sensor of the nutritional status of plants, and thus invertase plays a fundamental role in controlling cell differentiation and development (Sturm and Tang, 1999). Compositional changes observed in several developing fruits are associated with the changes in enzyme activity relating to sucrose metabolism (Ranwala *et al.,* 1991).

In plant intact tissue in the case of injury β-fructofuranosidase are rapidly formed in response to wounding and bacterial infection, suggesting their possible role in pathogen defense mechanism (Matsushita and Uritani, 1974). Commercially, invertase is mainly used in the confectionery industry, where fructose is preferred over sucrose because of a sweeter taste and a lower propensity to crystallize. Brewing, distilling, wine making and the baking of bread all requires freshly grown, specially developed yeast strains to inoculate fermentation. The activity of invertase determines the physical and organoleptic properties of bread (Antuna and Martinez-Anaya, 1993). As described above, the work on invertase is mainly focused on purification, physico-chemical characterization and structural stability and very few studies on

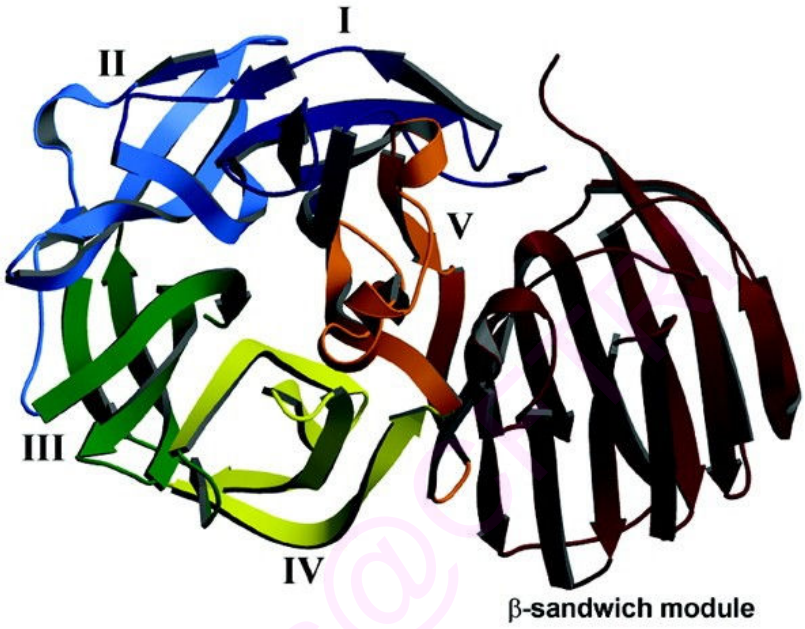

Fig. 1: Ribbon representation of the monomeric unit of *T. maritime* invertase, highlighting the N-terminal β-propeller module, the five blades (numbered I-V), and the C-terminal β-sandwich module.

[Courtesy: Reproduced from Alberto *et al.*, 2004]

stability of invertase in presence of cosolvents. But to date, there is not much information available on the effect of cosolvent as additives on the structure-function, kinetics, thermal stability, structure-stability relationship and preferential interaction parameter of invertase.

1.5. Nuclease P1

Nuclease P1 from *Penicillium citrinum* (EC 3.1.30.1) is a 36 kDa, single strand specific nuclease, consisting of 270 amino acid residues uses three zinc ions for both its phosphodiesterase and 3'-phosphomonoesterase enzymatic activities (Fujimoto *et al.,* 1974a; Maekawa *et al.,* 1991; Volbeda *et al.,* 1991). It contains two disulfide bonds and four carbohydrate attachment sites (Maekawa *et al.,* 1991). Nuclease P1 prefers single-stranded DNA and RNA substrates and hydrolyzes these completely, independent of the base sequence to 5'-mononucleotides (Fujimoto *et al.,* 1974b; Fujimoto *et al.,* 1974c; Fujimoto *et al.,* 1974d), but which are also capable of recognizing a range of non-A/non-B/non-Z conformations in double-stranded nucleic acids (Pulleyblank *et al.,* 1988). The enzyme is a phosphodiesterase, cleaving the bond between the 3'-hydroxyl and 5'-phosphoryl group of adjacent nucleotides and at the same time acts as a phosphomonoesterase removing the 3'-terminal phosphate group (Fujimoto *et al.,* 1974b). The UV absorption spectrum of nuclease P1 shows a maximum absorbance at 281 nm, with an extinction coefficient of 18.4 (Fujimoto *et al.,* 1975a), with a high amount of aromatic residues. From the molecular conformation the α-helix content of nuclease P1 is approximately 31% in its active form it contains three zinc ions per molecule (Fujimoto *et al.,* 1975b).

The structure of nuclease P1 was first solved for the tetragonal $P4_32_12$ crystal form, using multiple isomorphous replacements combined with anomalous scattering and solvent flattering (Volbeda *et al.,* 1991). One molecule of the enzyme has the longest dimension of about 57 Å (Fig. 2). The secondary structure consists mainly of helices that are arranged around the 31-residue-long C-terminal α-helix. Two disulfide bonds further stabilize the fold. Four carbohydrate attachment sites are found that involve asparagine side chains in Asn-X-Thr/Ser sequence motifs, and electron density for several ordered sugar residues has been observed. The three zinc ions of the active site are located at the bottom of a deep cleft, the walls of which are formed

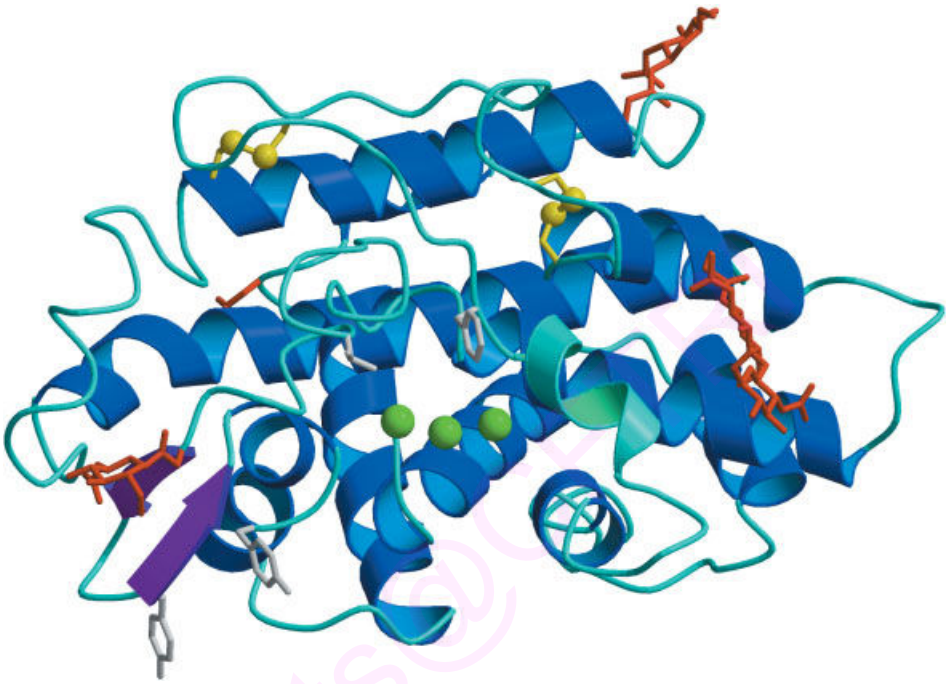

Fig. 2: Schematic representation of three dimensional structure of nuclease P1.
[Courtesy: Reproduced from Volbeda *et al.*, 2004]

mainly by secondary structure elements, including a 7-residue-long 3/10 helix and a β-hairpin (Volbeda *et al.,* 2004).

Substrate binding in nuclease P1 was studied by soaking the tetragonal crystals with single-stranded dithiophosphorylated R-stereoisomers of di-, tetra- and hexanucleotides as substrate analogues. These studies revealed the presence of two nucleotide-binding sites, one at the active site (Phe-site) close to the catalytic zinc and the second approximately 20 Å away at the periphery of the molecule. At both sites the base recognition involved stacking interactions with exposed aromatic residues as well as hydrogen bonding contacts with the carboxylate groups. Thus interaction occurred with Phe 61 and Val 132 at the first binding site and with Tyr 144 and Tyr 155 at the second site (Romier *et al.,* 1998).

Nuclease P1 and related enzyme have been extensively used by molecular biologists as analytical tool for the determination of nucleic acid structure, owing to their ability to recognize single-stranded nucleic acids and a wide variety of nucleic acid structure as well as structure variations. They completely hydrolyze single-stranded DNA and RNA substrates into 5'-mononucleotides. In addition, they may be used to detect non-A and non-B conformations in double-stranded DNA structures which unlike regular A- and B-DNA structure, are susceptible to enzymatic cleavage (Desai and Shankar, 2003; Pulleyblank *et al.,* 1988; Volbeda *et al.,* 2004). Nuclease P1 has been used for the isolation of eukaryotic mRNA cap structure (Furuichi *et al.,* 1975), base composition analysis of nucleic acids, removal of nucleic acids during protein purification (Zabriskie and DiPaolo, 1988), sequence analysis of end-group-labeled RNA and analysis of tRNA structure (Aultman and Chang, 1982).

Several physiological roles have been proposed, in trypanasomatid parasites which are incapable of purine biosynthesis, external membrane-bound P1-like nucleases are probably involved in the salvage of purines from host RNA and DNA (Debrabant *et al.,* 1995). In plants nuclease enzymes are thought to degrade RNA and single-stranded DNA substrates during several developmental processes, including senescence and programmed cell death (Aoyagi *et al.,* 1998). Monosodium glutamate has long been used as a food flavoring agent. However, the realization that the addition of an equimolar mixture of 5'-GMP and 5'-IMP to monosodium glutamate

can significantly increase the flavor enhancing capacity which led to considerable interest in the production of 5'-mononucleotides. Nuclease P1 which degrades RNA to 5'-mononucleotides, has been used for the industrial production of 5'-mononucleotides from yeast RNA (Kuninaka *et al.,* 1961).

Since their discovery, three decades ago the utility of nuclease P1 as an analytical tool has been widely recognized, due to its ability to recognize a wide variety of nucleic acid structures. The majority of the works have focused on physicochemical characterization along with mode of action. But to date, very little work is done on the stability in different solvents, such as effect of different stabilizing agents on structure-function relationship along with kinetics, thermal and pH stability of nuclease P1.

1.6. Lipases

Lipids constitute a large part of the earth's biomass, and lipolytic enzymes play an important role in the turnover of these water-insoluble compounds. Lipolytic enzymes are involved in the breakdown and thus in the mobilization of lipids within the cells of individual organisms as well as in the transfer of lipids from one organism to another (Beisson *et al.,* 2000).

Lipases (triacylglycerol ester hydrolases EC 3.1.1.3) are enzymes that catalyse the hydrolysis of the ester bonds of triacylglycerols. Lipases are found in all species of the animal kingdom as well as in plants and microorganisms such as yeast, bacteria and fungi. The increasing interest in lipase research over the past decades has likely occurred for three reasons. The first is related to the molecular basis of the enzyme catalytic function or the lipase paradigm. Indeed, lipases though water soluble, catalyze reactions involving insoluble lipid substrates at the lipid-water interface. This capability is due to the unique structural characteristic of lipases. The second reason is linked to the enzyme's medical relevance, particularly in atherosclerosis and hyperlipidemia and its importance in regulation and metabolism, since products of lipolysis such as free fatty acids and diacylglycerols play many critical roles, especially as mediators in cell activation and signal transduction. Later, it was discovered that lipases are powerful tools for catalyzing not only hydrolysis, but also

various reverse reactions such as esterification, transesterification, aminolysis in organic solvents (Villeneuve *et al.,* 2000).

In plants, the regulation in some cases the location and the exact physiological roles of lipases are not very clear. They are known to play an essential role in the mobilization of seed-storage lipids to support germination and post-germinative embryonal growth (Muto and Beevers, 1974). All the members of the lipase gene family have a conserved Ser, which is the nucleophile essential for catalysis. The active site triad of lipases consisting of Ser-His-Asp/Glu is reminiscent of the serine proteases (Blow, 1990). The present work will focus on structure-function, kinetics and activity studies on rice bran lipase and structure-function and stability studies of porcine pancreatic lipase, which have been extensively investigated in terms of reaction mechanism.

1.6.1. Lipase from rice bran

The biological functions of lipase in plants are well documented. They are known to play an essential role in the mobilization of seed-storage lipids to support germination and post-germinative embryonal growth. Apart from this, lipases are equally important in the field of nutrition, oil and fat industry. In rice bran, lipolytic activity causes rancidity making the by product unsuitable for human consumption.

Lipases are generally considered to be absent in most dry seeds and are probably synthesized de novo after the germination. In case of rice, most of the lipase activities are present in the outer layers of the grain. Two soluble lipases have been purified from rice bran (both isoforms Lipase I and II). The isolation and purification of rice bran lipase (Lipase I) was given by Funtasu *et al.,* (1971) and Shastry and Raghavendra Rao, (1971). Funtasu *et al.,* (1971) purified the enzyme by selective ammonium sulfate precipitation, series of chromatographic techniques in the presence of calcium ions. The purified enzyme show a molecular mass of 40 kDa with a pH optimum of about 7.5 and shows a specific activity of 4-7 units/mg of protein. It is activated by calcium and cleaves preferentially fatty acids from the sn-1 and sn-3 positions of triacylglycerols. Shastry and Raghavendra Rao, (1971) purified a rice bran lipase by ammonium sulfate fractionation, ion exchange chromatography and

adsorption on calcium phosphate gel. The purified enzyme shows a molecular mass of 41 kDa. It hydrolyses long and short chain synthetic triglycerides and oils from the rice bran with more activities towards tributyrin. Lipase II was purified by Aizono *et al.,* (1976) with a molecular mass of 32 kDa, a pI of 9.1, and pH and temperature optimum of about 7.5 and 27°C.

RBL used in this study were purified according to the procedure of Rajeswara and Prakash (1995) with higher yields and activity. The enzyme shows an ultraviolet absorption maximum of 276 nm with pH and temperature of 7.5 and 30°C. The enzyme shows a specific activity of 18 units/mg of protein, it contains 274 amino acids with molecular weight of 30 kDa, with a secondary structural content of 15% α-helix, 51% β-sheet and the rest are aperiodic. The mechanism of action and substrate binding were similar to that of serine proteases. So far the studies on the rice bran lipase were mainly focused on purification and structure-stability of the enzyme. But no work has been done on the inhibitory activity of metal ions and plant based inhibitors such as azadirachtin on structure-function and structure-properties relationship of rice bran lipase.

1.6.2. Lipase from porcine pancreas

Pancreatic lipase plays a key role in dietary fat absorption in the intestine. It converts insoluble long chain triglycerides into more polar molecules that are able to cross the brush border membrane of enterocytes as mixed micelles with bile salts (Borgstorm and Erlanson, 1973).

Porcine pancreatic lipase is a glycoprotein composed of 450 amino acids with a calculated molecular weight of 50 kDa. The enzyme consists of six disulfide bridges, Cys 4-Cys 10, Cys 91-Cys 102, Cys 238-Cys 262, Cys 286-Cys 297, Cys 300-Cys 305 and Cys 434-Cys 450, with two free cysteines, Cys 104 and Cys 182, the enzyme is N-glycosylated at Asn 167 (De Caro *et al.,* 1981; Fournet *et al.,* 1987; Hermoso *et al.,* 1996). The UV absorption spectrum of porcine pancreatic lipase shows a maximum absorbance at 280 nm, with an extinction coefficient of 13.3 (Donner *et al.,* 1976). The porcine pancreatic lipase is a two domain structure (Fig. 3), the larger N-terminal domain (residues 1-336) has a α/β structure, containing the

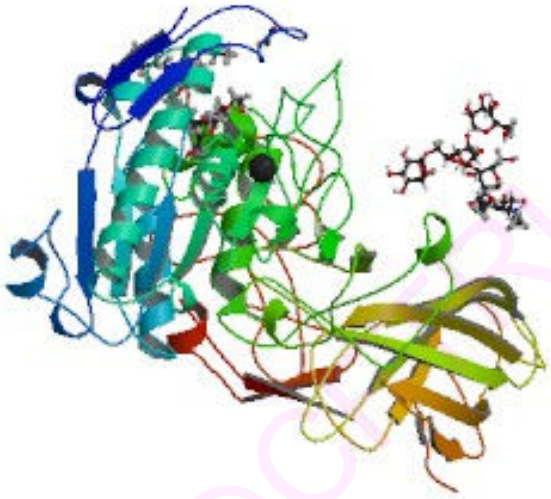

Fig. 3: Schematic ribbon diagram of the porcine lipase-colipase structure. [Courtesy: Reproduced from protein data bank access code 1ETH]

catalytic triad; Ser 153, Asp 177 and His 264. The C-terminal domain (residues 337-450) assumes a β-sandwich, and contains the binding site for colipase (Hermoso *et al.,* 1996). From the secondary structure determination the α-helix content is approximately 23% (Hermoso *et al.,* 1996). Unlike most of the pancreatic enzymes which are secreted as proenzymes and further activated by proteolytic cleavage in the small intestine, pancreatic lipases are directly secreted as a 50-kDa active enzyme.

Recent structural analyses have been shown conclusively that lipases contain the same constellation of the catalytic triad, Ser-His-Asp, present in all serine proteases, although the topological position of the individual residues side chain varies (Brady *et al.,* 1990). The lipase-catalyzed hydrolysis of monomeric substrates involves the Ser-His-Asp triad and the formation of an acylenzyme.

Lipolytic enzymes are currently attracting an enormous attention because of their biotechnological potential (Benjamin and Pandey, 1998). They constitute the most important group of biocatalysts used in various sectors, as pharmaceutical, bioconversion of surplus fats and oils into higher value products for food industrial uses, surfactants, oleochemistry and detergency industry (Villeneuve *et al.,* 2000). Lipases are also capable of catalyzing the reverse reaction, achieving esterification, transesterification (acidolysis, interesterification and alcoholysis), aminolysis, oximolysis and thiotransesterification in anhydrous organic solvents, biphasic systems and in micellar solution with chiral specificity, using hydrolases, oxidoreductases or lyases as biocatalysts for the synthesis of chiral drugs (Klibanov, 1989; Patel, 2003; Gotor-Fernandez *et al.,* 2006).

Due to their ability to involve in large number of catalysts, considerable efforts were made to understand the structural stability of lipase from porcine pancreas and the role of stabilizing additives are scarce. But to date, there is not much information available on the role of cosolvents additives on structure stability, preferential interaction parameter along with thermal stability of lipase from porcine pancreas.

Based on the above literature cited, an in depth study of the effect of cosolvents on the stability of a set of three well characterized enzymes belonging to hydrolases (nuclease P1, invertase, and lipases) at varying cosolvent concentrations, temperature and pH values and interaction studies with rice bran lipase using selenium, lithium and azadirachtin is undertaken in this investigation. A detailed study of stabilization of invertase, nuclease P1 and porcine pancreatic lipase with stabilizing cosolvents such as glycerol, xylitol, sorbitol, sucrose and trehalose has been undertaken to understand the mechanism of cosolvent mediated stability of enzyme in detail from the point of view of activity, thermal and pH stability, kinetics and structural stability as related to structure-function and structure-activity relationship of the enzymes with cosolvents. In the present study the effect of urea and GuHCl on the conformation and stability of nuclease P1 has also been undertaken to gain insight into the mechanism of stability of nuclease P1 in low concentrations of urea and GuHCl from the point of view of activity, kinetics and structural stability to understand its structure-function correlation and potential applications. The interaction of various ligands such as metal ions and azadirachtin on the structural and enzymatic activities of rice bran lipase are also investigated with a view of understanding the stabilization of rice bran lipase and also to understand the mechanism of interaction of these ligands on enzymes for understanding the structure-function and stability phenomenon.

SCOPE AND OBJECTIVES

SCOPE AND OBJECTIVES

The functionality of a given protein is determined by its unique molecular structure and interactions with the surrounding condition where it is present. In their natural environment, proteins tend to adopt particular molecular conformations that determine their properties. When proteins are extracted from their natural condition the main focus generally is to retention of the activity. The stability of proteins against changes in the thermodynamic as well as in the chemical conditions of the solvent is only marginal. Most enzymes can be easily inactivated by a variety of conditions such as temperature, changes in pH or polarity of the medium or the presence of chaotropic agents. This phenomenon is directly related to the loss of catalytic activity and this becomes a limiting factor for the use of enzymes in biotechnological processes.

Considerable effort has been devoted in nearly all fields of biochemical sciences to improve the protein stability. The stability of proteins is governed by the intramolecular interactions of protein functional groups and their interaction with solvent medium. This can be attributed to the unique properties displayed by water as a solvent system and it can be modified by addition of cosolvent molecules. This in turn alters the water structure surrounding the protein molecules. Cosolvents can affect their aqueous environments in numerous ways such as, altering viscosity, density, dielectric constant, refractive index, surface tension, pH and osmotic pressure.

Cosolvents can be divided into four categories depending on their effect on protein transitions as neutral, favorable, unfavorable and combined. The neutral and combined cosolvents are rather rare in real-life situation. Favorable and unfavorable cosolvents play a very major role in protein stability. Favorable cosolvents oppose a protein transition; the mechanism is based on the fact that the cosolvents act as a stabilizer because the change in transfer free energy for the respective transition is positive. Sugars (sucrose and trehalose) and polyols (glycerol, xylitol, sorbitol etc.,) fall into the category of stabilizing agents. Destabilizing agents favor a protein transitions, and they are believed to be favoring the unfolded state by exposing more surface area of proteins, Urea, GuHCl and metal ions such as selenium and lithium

fall into these categories along with some plant based secondary metabolites such as azadirachtin.

In order to understand the effects of stabilizing/destabilizing agents on the stability, structure-function relationship and kinetics of enzymes, three different class of enzymes is selected for our study; these are invertase from *Candida utilis*, nuclease P1 from *Penicillium citrinum*, lipase from rice bran and lipase from porcine pancreas.

Invertase known as β-fructofuranosidase catalyzes the hydrolytic breakdown of sucrose to glucose and fructose and widely distributed in microbes plant and animals. It is extensively used in confectioneries, fermentation of cane molasses into ethanol and in calf feed preparation. Enhancement of the stability and activity of the enzyme will expand its use over a wide range of temperature and pH. Here the cosolvents have been used to stabilize the enzyme and to enhance the activity which exploits new industrial potentials of invertase.

Nuclease P1 from *Penicillium citrinum* cleaves phosphodiesters bonds specifically in single stranded nucleic acids. This enzyme is widely used in molecular biology as an analytical tool for the determination of nucleic acid structure. Nuclease P1 has been used in various food industries to produce 5'-nucleotides, which is a flavor enhancing agent. For wide application of this enzyme across the various food and pharmaceutical industries the structure-function and stability relationship must be established. The stability of the enzyme plays crucial role during industrial process. The enhancement of activity and stability of this enzyme in presence of cosolvents have not been studied in detail. Studies on the behavior of nuclease P1 and its interaction with cosolvents under various conditions of temperature and pH forms one of the major objectives of this study.

Rice bran lipase produces free fatty acids leading to the rancidity in rice bran. Further, inactivation of rice bran lipase is an important role in order to produce edible grade oil devoid of free fatty acids. In this study various ligands such as selenium, lithium and azadirachtin were used to inactivate the enzyme.

Porcine pancreatic lipase plays an important role in chemical and pharmaceutical industries owing to the usefulness in both hydrolytic and synthetic reactions. Extreme condition of pH and temperature lead to the instability of the lipase which limits its industrial application. The structure-function and stability relationship of lipase from porcine pancreas is poorly known in presence of cosolvents and these studies can reveal a detailed mechanism for the stabilization of the enzyme using various cosolvents.

In order to understand the exact relationship between the structure and function of these enzymes in presence of cosolvents and ligands have been investigated. Thus the detailed elucidation of the mechanism responsible for stabilization and destabilization of these enzymes is the focus of this investigation.

Specific objectives of the present study are:

Chapter 1. Studies on invertase

1. *Effect of cosolvents on the activity, stability, structure-function, kinetics and preferential interaction studies of invertase.*

   The effect of selected cosolvents such as glycerol, xylitol and sorbitol on the activity and stability of invertase was studied by activity measurements at different conditions with different concentrations of above cosolvents. The structural and kinetics of cosolvent mediated stability in different condition were also studied by different spectrophotometric techniques. The preferential interaction parameters of invertase in presence of above cosolvents were studied to correlate stability.

Chapter 2. Studies on nuclease P1

2a. *Effect of cosolvents on the activity, stability, structure-function and kinetic studies of nuclease P1.*

   The stability of nuclease P1 in presence of various cosolvents like glycerol, sucrose and trehalose were monitored by activity measurements at different conditions with different concentrations. Kinetics of native and cosolvent treated

nuclease P1 at different conditions was measured; spectral and conformational changes of nuclease P1 with cosolvents were also evaluated by different spectrophotometric methods.

2b. *Effect of denaturants on the activity, stability, structure-function and kinetics of nuclease P1.*

Effect of low concentration of denaturants on the activity and conformational stability of nuclease P1 was monitored by kinetics, ultraviolet circular dichroic spectroscopy, fluorescence spectroscopy and thermal denatuartion temperature analysis to correlate the stability of the enzymes in presence of denaturants.

Chapter 3. Studies on lipases

3a. *Effect of selected ligands such as metal ions and azadirachtin on the inhibitory effect on rice bran lipase.*

Activity, kinetics and stability of native and ligands such as metal ions and azadirachtin treated rice bran lipase was measured by evaluation of activity, structural integrity and conformation as a result of modification of the rice bran lipase by ligands such as metal ions and azadirachtin.

3b. *Effect of cosolvents on the activity, stability, structure-function, kinetics and preferential interaction studies of porcine pancreatic lipase.*

Enzyme activity, thermal and pH stability of porcine pancreatic lipase in presence of selected cosolvents (glycerol, xylitol, sorbitol, sucrose and trehalose) were monitored by activity measurements at different conditions with different concentrations. Cosolvents mediated mechanism of stabilization was measured by kinetic approach; spectral changes, thermal and pH stability of porcine pancreatic lipase in presence of above cosolvents were also monitored by ultraviolet circular dichroic spectroscopy, fluorescence spectroscopy and thermal denatuartion temperature analysis; preferential interaction parameter of porcine pancreatic lipase in cosolvents as related to assess the relationship between thermal stability and relative hydration of the enzyme in presence of cosolvents.

Hence, the objectives of the present study as indicated above and the data generated would give more insight into understanding the mechanism of activity, structure-function and structural stability of invertase, nuclease P1 and lipases in presence and absence of selected cosolvents and ligands.

MATERIALS AND METHODS

MATERIALS AND METHODS

1. Materials

Invertase grade X from *Candida utilis*, nuclease P1 from *Penicillium citrinum*, Lipase type-VI-S from porcine pancreas, D-sorbitol, sucrose, glycerol, xylitol, D-trehalose, urea, guanidine hydrochloride, gum arabica, sodium taurocholate dihydrate, tributyrin, 3,5-dinitro salicylic acid, sodium dodecyl sulfate, bisacrylamide, N-bromosuccinamide, bovine serum albumin, calcium chloride dihydrate, glycine, acrylamide, 2-mercaptoethanol, Bradford reagent, standard reference buffers, colipase, zinc chloride, Sepharose CL-6B, Sephadex G-75, triacetin, azadirachtin, ribonucleic acid from *Tarula utilis*, calf-thymus DNA, $CuSO_4$, $FeCl_2$ and $CoCl_2$, molecular weight markers were purchased from Sigma-Aldrich chemical company, St Louis, MO, USA. Triton X-100 was obtained from Beckman Inc., USA. Spectrapor molecular porous membrane tubing was from Spectrum Laboratories, Inc, Rancho Dominguez, CA, USA. Blue dextran, Superdex-75, Sephadex G-25 were purchased from Pharmacia fine chemicals, AB, Upasala, Sweden. Coomassie brilliant blue G-250, coomassie brilliant blue R-250 was from Bio-Rad Laboratories, Richmond, USA. N,N,N,N,tetramethyl ethylenediamine, ammonium persulfate, bromophenol blue were from Research Organics Inc, Cleveland, Ohio, USA. Methanol, acetic acid glacial, sodium acetate, sodium chloride, glycine, Tris buffer, potassium chloride, ammonium sulfate, trichloroacetic acid, potassium sodium tartrate tetrahydrate, sulfuric acid, hydrochloric acid, sodium hydroxide, di-phosphorous pentaoxide, tris hydroxymethyl aminomethane, dichloromethane, vanillin were procured from E. Merck (India) Ltd, Mumbai, India. Di-sodium hydrogen phosphate, sodium dihydrogen phosphate was from Hi-Media laboratories Pvt. Ltd., Mumbai, India. Folin & ciocalteu's phenol reagent was procured from Sisco Research laboratories Pvt. Ltd., Mumbai, India. Selenium dioxide, lithium sulfate were procured from Loba Chemie Pvt. Ltd., Bombay, India. All the chemicals used in the study were of the analytical grade. Quartz triple distilled water was used in all the experiments.

2. Methods

2.1. Preparation of enzymes

Invertase, nuclease P1, lipase from rice bran and lipase from porcine pancreas were procured or prepared in the laboratory as described below and subjected to dialysis against quartz triple distilled water for 24 hour at 4°C and lyophilized. The homogeneity of the enzymes was evaluated by SDS-PAGE and isoelectric focussing measurements.

2.2. Isolation and purification of rice bran lipase

Fresh rice bran (*Oryza sativa* var. Jaya) collected from local rice mills was used for the isolation of rice bran lipase (RBL). RBL was isolated and purified according to the modified procedure of Rajeshwara and Prakash (1995). The total protein extracts from the defatted rice bran flour was subjected to selective ammonium sulfate precipitation. The pellet obtained was suspended in 0.05 M Tris-HCl buffer (pH 7.5) and was dialyzed against six volumes of buffer at 4°C. The clear supernatant obtained after centrifugation (6000 rpm for 20 min) was loaded onto a pre-equilibrated DEAE-Sepharose CL-6B anion exchange column. The enzyme fraction eluted at 0.2 M KCl gradient was pooled and further subjected to 55% ammonium sulfate precipitation and dialyzed. The clear solution was then loaded onto Sephadex G-75 column pre-equilibrated with 0.05 M sodium phosphate buffer (pH 7.4). The enzyme fraction was pooled, concentrated, dialyzed and lyophilized. The enzyme thus obtained was stored at −20°C in a deep freezer and used for further studies (Rajeshwara and Prakash, 1995).

2.3. Isolation of azadirachtin from neem seed kernel

Azadirachtin from fresh neem seed (*Azadirachta indica*) was isolated and purified by modified procedure of Dai *et al.,* (1999). The total azadirachtin from defatted neem seed kernel was extracted with ethanol by stirring at room temperature for 4-6 hours. The extract was evaporated under vacuum. The product was dissolved in dichloromethane for the azadirachtin assay.

2.4. Preparation of dialysis membrane

The 23 mm flat width dialysis membrane of 6000-8000 molecular weight cut-off was washed with distilled water thoroughly. The membrane was further treated with 0.2% sodium bicarbonate and 0.2% EDTA and boiled for 30 minutes. The treated bags were rinsed thoroughly and boiled thrice in quartz triple distilled water before using in different dialysis experiments (Muralidhara, 1997).

2.5. Freeze drying

The protein after the dialysis was freeze dried in Virtis freeze dryer. Freeze drying was continued till the complete removal of moisture from the protein samples. Protein were sealed in airtight containers and stored in desiccator. These freeze dried protein samples were used for further studies.

2.6. Methods of protein estimations

The protein concentrations were measured by using spectrophotometric, Folin Ciocalteu's phenol reagent and Bradford methods as described below under each method.

2.6.1. Protein concentration determination using extinction coefficient

The protein concentration was determined spectrophotometrically using extinction coefficient ($E^{1\%}_{1cm}$) of 1.8 at 278 nm for invertase (Iizuka and Yamamoto, 1972), 18.4 at 281 nm for nuclease P1 (Fujimoto *et al.,* 1975a), 15.25 at 276 nm for rice bran lipase (Rajeshwara *et al.,* 1996) and 13.3 at 280 nm for porcine pancreatic lipase (Donner *et al.,* 1976) in a Shimadzu UV-1601 double beam UV-Visible spectrophotometer.

2.6.2. Protein estimation by Folin & Ciocalteu's phenol reagent method

The protein content of the enzymes was measured according to the procedure of Lowry's (Lowry *et al.,* 1951). The reaction mixture consists of following reagents, Reagent A: 2% Na_2CO_3 in 0.1 N NaOH; Reagent B: 0.5% $CuSO_4$. $5H_2O$ in 1% sodium potassium tartarate; Reagent C: Mix 50 ml of reagent A with 1 ml of reagent B. Reagent D: Diluted Folins reagent. To a sample of 5 to 100 µg of protein in 0.2 ml or less in a 3 to 10 ml test tube, 5 ml of reagent C is added. Mix well and allowed to

stand for 10 min. 0.5 ml of reagent D was added, mixed rapidly and allowed to stand for 30 min. The absorbance of the solution was recorded at 660 nm and concentration of protein was determined using BSA standard curve (Lowry *et al.,* 1951).

2.6.3. Protein estimation by Coomassie Blue (Bradford) method

The protein in the solution was estimated by the method of Bradford (1976). A Bradford stock solution consists of 200 ml 88% phosphoric acid, 350 mg serva blue G were prepared in 95% ethanol and stored for further use. The working solutions consists of 425 ml distilled water, 15 ml 95% ethanol, 30 ml 88% phosphoric acid and 30 ml Bradford stock solution. All the solutions were mixed together and filtered through Whatman No. 1 filter paper and stored in brown glass bottles. 1 ml of Bradford working solutions was added to 100 µl of protein solution mixed well and allowed to stand for 2 min. Then absorbance was recorded at 595 nm. The protein concentration was estimated using a standard curve of BSA (Bradford, 1976).

2.7. Colorimetric determination of total azadirachtin

Total azadirachtin in neem seed kernel was estimated according to the procedure of Dai *et al.,* (1999). The neem seed extract obtained from the ethanol extraction was concentrated under vacuum. The concentrate was dissolved in dichloromethane. 0.01-0.10 mg/ml standard azadirachtin solution were made in dichloromethane. 0.7 ml of standard azadirachtin or neem seed extract of unknown concentration were added in 0.2 ml of methanol containing 0.02 mg/ml of vanillin. After shaking manually, the mixture was left at room temperature for 2 min. 0.3 ml of concentrated sulfuric acid was added in three portions (0.1 ml each) and the mixture was stirred for 10 sec after each addition. After the addition of sulfuric acid was completed, 0.7 ml of methanol was added to convert the two-layer mixture into a homogeneous solution that instantly developed a blue-green color. The solution was kept at room temperature for 5 min before the absorbance were measured at 577 nm. The blank solution was substituting the test solution with an equal volume of dichloromethane in the above mixture. Total azadirachtin in the neem seed extract were quantified using a calibration curve generated from standard azadirachtin solutions in dichloromethane (0.01-0.10 mg/ml) using the above procedure.

2.8. Oil extraction and free fatty acid determination in rice bran

The total oil in the fresh rice bran and those treated were determined by Soxhlet extraction method (AOAC, 1995). About 5 gm of fresh and treated rice bran was taken in a Whatman thimble. The thimble was placed in a Soxhlet extraction unit. Hexane AR grade was used as the extraction solvent. Extraction was carried out by heating the Soxhlet unit. After the extraction, the preweighed receiver flask containing the extracted fat was dried initially on a water bath at 98-100°C and then using an oven at 100 ± 2°C till constant weight was obtained. Known weight of the oil was taken in a flask and the acid value was determined as explained in (AOCS, 1997). The percentage FFA was calculated as oleic acid.

2.9. Size exclusion chromatography using fast protein liquid chromatography (FPLC)

Size exclusion chromatography of the native and ligands treated RBL was carried out on Superdex-75 column using FPLC. The RBL (1.4×10^{-5} M) was incubated in the presence of different concentration of ligands at 30°C for 30 min in Queue orbital shaker with gentle stirring. Both native and ligands treated samples were dialyzed with 3 or 4 changes against respective buffers and centrifuged at 10000×g for 10 min. Clear supernatant was loaded onto Superdex-75 column. The protein was eluted at a flow rate of 0.5 ml/min in a 60 min run. Blanks containing respective concentration of ligands were also run to give necessary corrections.

2.10. Electrophoretic methods

2.10.1. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out using discontinuous buffer system as described by Laemmlli (1970). The polyacrylamide gel (10-12.5% T) containing 0.1% SDS were cast in 1.5 mm slab gel apparatus. The electrophoresis was carried out in 0.192 M glycine, 0.025 M Tris, 0.1% SDS as electrode buffer at constant voltage of 100 mA. The protein (10-20 µg) was mixed with the sample buffer, pH 6.8 containing 5% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol, 2% (w/v) SDS and 0.1% bromophenol blue. Samples were heated in a boiling water bath for 5 min and subjected to electrophoresis. The SDS-PAGE molecular weight markers were also treated in

similar manner and electrophoresed. After the electrophoresis, the gels were stained with Coomassie Brilliant Blue R-250 staining solution and destained. The Staining solution was prepared by dissolving 1 mg/ml of Coomassie Brilliant Blue R-250 in a solution of methanol and glacial acetic acid (destaining solution). Gel was immersed in 200 ml of staining solution and shaken on gel rocker for 30 min. The destaining solution consists of 25% methanol and 5% glacial acetic acid. Gel was destained with destaining solution until desired back ground is achieved.

2.10.2. Isoelectric focusing

Isoelectric focusing of lipase, nuclease P1 and invertase was determined according to the method of O'Farrel (1975). Proteins were focused on a commercially available Ampholine PAGplate of a 1 mm thick polyacrylamide gel cast on a plastic support film. Ampholine PAGplate with a pH range of 3.5-9.5, was used in the isoelectrofocussing. The gel matrix contains polyacrylamide T = 5%, C = 3%, anode solution (1M H_3PO_4), cathode solution (1M NaOH). The fixing solution contains 29g TCA, 8.5g sulphosalicylic acid in 250 ml water. Multiphor II electrophoresis unit was connected with the MultiTemp II thermostatic circulator and set the temperature at 10°C. The electrode strips were soaked evenly in the appropriate electrode solution and applied along the edges of the isoelectrofocussing gel. The samples of about 0.1-0.2 mg of proteins are loaded and the running condition was 600 Voltage, 15 mA current, 10W power for 1.5 hour. The gel was placed in a fixing solution for about 30 minutes and after fixing washed with destaining solution. There after the gels were stained with Coomassie Brilliant Blue R-250 staining solution and destained as described earlier.

2.11. Determination of various enzyme activities

2.11.1. Invertase assay

Invertase activity was determined based on the method described by Miller *et al.,* (1960) using sucrose as substrate. The assay mixture (1 ml) containing 100 mM sucrose and 10 µl (0.1-0.2 mg/ml) of invertase incubated for 15 min at 55°C in 0.05 M acetate buffer, pH 4.5. The enzymatic reaction was terminated by addition of 2 ml DNS reagent followed by heating in a boiling water bath for 5 min. Solution was diluted with 7 ml water, mixed well and absorbance was measured at 540 nm. The

amount of glucose or fructose released were measured using dinitrosalicylic acid (DNS) method (Miller *et al.,* 1960). One unit of activity is defined as the amount of enzyme that hydrolyzes 1 µmole of sucrose per min under the assay condition. Glucose was used as a standard in the calibration curve (Miller *et al.,* 1960).

2.11.2. Nuclease P1 assay

Nuclease P1 activity was measured using RNA as a substrate by the method of Fujimoto *et al.,* (1974a) with slight modification using lead acetate by replacing uranyl acetate (Zhu *et al.,* 1996). The nuclease P1 activity was measured in terms of the amount of acid-soluble nucleotides produced by RNA hydrolysis which is catalyzed by nuclease P1. The reaction mixture consisting of 0.2 ml of acetate buffer, pH 5.3, 0.2 ml of RNA solution (2 mg/ml stock) and 3.5 µg of the enzyme (0.1 mg/ml) solution were incubated at 37°C for 15 min. The reaction was stopped by adding 0.5 ml of ice cold lead acetate reagent (0.25% w/v), allowed to stand for 20 min in ice bath. The precipitate formed was removed by centrifugation at 5000 × g for 15 min. The supernatant fluid was diluted 8-fold with water and the absorbance was measured at 260 nm against a blank incubated without enzyme. The amounts of acid soluble products were calculated by assuming a molar extinction coefficient of 10,600 for RNA hydrolyzates. To see the effect of DNA as substrate, nuclease P1 activity measurements were determined using calf thymus DNA, which is a double stranded nucleic acid. Double-stranded calf thymus DNA was dissolved (2mg/ml) in buffer, boiled for 10 min and placed on ice immediately to prevent the renaturation. The remaining assay procedure was followed in the same manner as described for RNA as above (Fujimoto *et al.,* 1974a).

$$\text{Units/mg solid} = \frac{\Delta A_{260\ nm} \times \text{Dilution factor}}{\text{Incubation time} \times 10600 \times \text{mg of sample used}} \quad \text{-------- (1)}$$

one unit of nuclease P1 activity was defined as formation of 1.0 µmole of acid soluble nucleotides from RNA per min, pH 5.3 at 37°C.

2.11.3. Effect of detergent and metal ions on nuclease P1 activity

To examine the effect of detergent on the nuclease P1 activity, Triton X-100, was added to the enzyme at a final concentration of 1 and 2% (w/v) respectively. The

nuclease P1 assay was performed to determine the activity. To study the effect of metal ions on nuclease P1, the enzyme was pre-incubated with different concentrations of metal ions such as $CuSO_4$ and $CoCl_2$ in the range of 1×10^{-6} M to 1×10^{-3} M. Residual nuclease P1 activity was determined under standard assay condition.

2.11.4. Lipase assay

Lipase activity was determined based on titrimetric method in Mettler Toledo DL-12 titrator. Tributyrin was used as substrate for porcine pancreatic lipase and activity was measured at 37°C (Thomas *et al.,* 2005). Triacitin was used as substrate for rice bran lipase and activity was carried out at 30°C (Sudhindra Rao *et al.,* 1991). An enzyme solution along with cosolvents was incubated for one hour with 4 ml solution of 0.11 M emulsified tributyrin in 10 mM Tris-HCl buffer containing 0.1 M NaCl and 5 mM $CaCl_2$. The reaction mixture consists of 4 ml of substrate, 10 µl enzyme (0.7 mg/ml) along with different concentrations of cosolvents. The reaction mixture was incubated in a Queue orbital shaker at 37°C at 100 rpm for lipase from porcine pancreas. For rice bran lipase, 5% (w/v) solution of triacetin containing 0.1% triton X-100 was prepared in 0.05 M sodium phosphate buffer at pH 7.4. The reaction mixture containing 4 ml substrate, 2 ml enzyme (0.5-2 mg/ml), and 10 µl of 0.1 M $CaCl_2$ was incubated in a Queue orbital shaker at 30°C. Enzymatic reactions of both the lipases were terminated by the addition of 4 ml distilled alcohol. In the case of blank, enzyme was first inactivated by the addition of alcohol. The liberated acid was titrated against 0.05 N alkali to an end point of pH 9.5. One unit of enzyme activity was expressed as micro equivalents of alkali consumed per milligram of protein per hour of incubation, according to the equation

$$\text{Specific activity} = \frac{(V_2 - V_1) \text{ x N of alkali x } 1000}{\text{a x incubation period}} \qquad \text{-------- (2)}$$

where, V_1 and V_2 are the volumes of standard alkali (NaOH) consumed by blanks and sample respectively, N is the normality of the standard alkali, ‘a’ is the concentration of protein in the reaction mixture expressed in mg/ml, and 1000 is the factor to express the activity in micro equivalent of alkali consumed (Sudhindra Rao *et al.,* 1991).

2.12. Reversibility studies

2.12.1. Dialysis method

One ml of enzymes were incubated with different concentration of cosolvents in different condition and dialyzed against respective buffers for 36 hours at desired temperature of incubation. Proper blanks of enzyme solution alone in the dialysis bags were used to monitor the reaction.

2.12.2. Gel filtration on Sephadex G-25 column

One ml of enzyme solutions was incubated with different concentration of cosolvents in different condition of temperature and pH. The enzyme was loaded on to Sephadex G-25 column (9 cm x 0.5 cm) and eluted with respective buffers. The eluted enzyme solutions were used for activity measurements. Proper blanks of enzyme solution alone in the column were used to monitor the reaction.

2.13. Analysis of kinetic data

2.13.1. Determination of kinetic constant of enzymes

Enzyme's kinetic parameters provide a measure of its catalytic efficiency. For kinetic studies enzyme was incubated with different concentrations of cosolvents and inhibitors in a defined conditions. The Michaelis-Menten constant (K_m) and the inhibition constant (K_i) of the enzyme were determined by employing the Lineweaver and Burk (1934) double reciprocal plot obtained from the initial velocity studies using the designated substrate and inhibitor concentrations for measuring the reaction rates (Allison and Purich, 1979).

2.13.2. Determination of catalytic constant of enzymes

The catalytic constant (k_{cat}) of the enzymes was determined according to the following method (Segel, 1975). This quantity is also known as the turnover number of an enzyme. This can be defined as 'number of moles of substrates transformed per minute per mole of enzyme (units per micromole of enzyme) under optimum conditions'. From the kinetic and catalytic constant values apparent second-order rate constant (k_{cat}/K_m) of the enzymes was calculated. The quantity k_{cat}/K_m is therefore a measure of an enzyme's catalytic efficiency (Voet and Voet, 1996).

2.14. Spectroscopic methods

2.14.1. Ultraviolet absorption spectra

Ultra violet absorption spectra of enzymes were routinely recorded with Shimadzu UV 1601 (Shimadzu, Japan) UV-Visible double beam spectrophotometer in the range of 200-350 nm. Clear protein solutions with an absorbance of 0.4-0.5 were used in all spectrophotometric readings using quartz cuvettes.

2.14.2. Measurement of extinction coefficient in different solutions

The extinction coefficient of enzymes varies with the third component. To obtain the $E^{1\%}$ in presence of third component, identical aliquots of native enzyme stock solution were mixed volumetrically to identical extents with native buffer as well as mixed solvents. The ultraviolet difference spectra were recorded in Shimadzu UV 1601 UV-Visible double beam spectrophotometer. From the ratio (A_{native}/A_{mixed} solvent) with corresponding blanks it is possible to calculate the $E^{1\%}$ of enzymes in mixed solvents.

2.14.3. Circular Dichroic spectral measurements

Far UV-Circular dichroic spectral measurements of the enzymes were carried out using an automatic recording Jasco J-810 spectropolarimeter fitted with a xenon lamp and calibrated with +d-10-camphor sulphonic acid, using a protein concentration of 8.9×10^{-7} M, 6.9×10^{-6} M, 9.6×10^{-6} M and 3.8×10^{-6} M for invertase, nuclease P1, rice bran lipase and porcine pancreatic lipase respectively. The spectra were recorded in the wavelength range of 195-260 nm with 0.1 nm increments using path length of 1 mm for far-UV regions. The scan speed was 10 nm/min using a bandwidth of 1 nm. The spectra were taken as an average of three scans. Dry nitrogen was purged continuously before and during the experiment. All the measurements were made at 25°C. The enzyme solution was prepared in respective buffers in presence and absence of different cosolvents. Mean residue ellipticity values were calculated using a value of 115 for mean residue weight. The molar ellipticity values were obtained at 1 nm interval by using the equation (Alder *et al.,* 1973).

$$\theta_{MRW} = \frac{\theta_{obs} \times MRW}{10 \times d \times C} \qquad \text{-------- (3)}$$

where, θ_{MRW} is the mean molar ellipticity per residue, $(\theta)_{obs}$ is observed ellipticity, d is the path length in cm, C is a protein concentration in g/ml and MRW is mean residue weight of the protein. The values obtained were normalized by subtracting the base line recorded for the buffer under similar conditions. The secondary structural analysis was done with the help of programme in the instrument (Yang *et al.,* 1986).

2.14.4. Fluorescence spectra

The fluorescence emission spectra of the proteins were recorded in Shimadzu RF-5000 spectrofluormeter (Shimadzu, Japan) in different conditions in presence and absence of cosolvents, denaturants and ligands. The enzymes were pre-incubated with cosolvents, denaturants, metal ions and azadirachtin. The protein concentrations were maintained as 3.6×10^{-7} M, 1.4×10^{-6} M, 2.6×10^{-6} M and 4.6×10^{-7} M for invertase, nuclease P1, rice bran lipase and porcine pancreatic lipase respectively. The excitation wavelengths were set at 281, 283 and 285 nm for invertase, lipases and nuclease P1 respectively. The emission spectra were recorded in presence and absence of different concentration of above solvents in the range of 300-400 nm with excitation and emission slit width of 5 nm.

2.14.5. Thermal denaturation studies

The effect of different concentrations of cosolvents on the thermal denaturation profile of invertase, nuclease P1 and porcine pancreatic lipase were studied using Varian Carry 100 Bio UV-Visible spectrophotometer using a scan rate of 1°C per min at 287 nm in the temperature range of 30-90°C. Respective buffers were used in the reference cell. Spectral data were stored and analyzed using specific software with the instrument. Apparent T_m was calculated from the absorbance. From the thermal denaturation profile, fraction of protein unfolded was calculated using a standard equation (Pace and Scholtz, 1997).

$$F_u = \frac{Y_F - Y}{Y_F - Y_U} \qquad \text{-------- (4)}$$

where, Y_F is the absorbance of protein solution in the native state, Y_U is the absorbance of protein solution in unfolded state, Y is the absorbance of the protein solution at different temperatures and F_u is the fraction unfolded. The apparent

thermal denaturation temperature T_m is defined as the temperature at which the value of Fu is 0.5. The results are average of three independent experiments.

2.15. Partial specific volume measurements

Density measurements were made by using Anton Paar DMA-5000 ultra precision densitymeter at 20.00 ± 0.02°C and the apparent partial specific volume, ϕ (ml/g) was calculated according to established procedures, using the equation (Casassa and Eisenberg, 1961; 1964).

$$\phi = 1 / \rho_0 [1 - (\rho - \rho_0) / C] \qquad \text{-------- (5)}$$

where, ϕ is the apparent partial specific volume, ρ and ρ_0 are the density of the solution and solvent respectively and C is the concentration of protein. The partial specific volume of protein was calculated by extrapolating the obtained values at different concentration of protein to zero protein concentration. The partial specific volume of proteins was obtained in isomolal (ϕ_2^0) and isopotential ($\phi`_2^0$) conditions, and the preferential interaction parameters were calculated using standard procedures (Lee *et al*., 1979; Prakash and Timasheff, 1985). The notation for 3 components was taken as water, protein and cosolvent as component 1, 2 and 3 respectively (Scatchard, 1946; Stockmayer, 1950).

In a typical three component systems the preferential interaction parameters (ξ_3) is calculated using the equation (Casassa and Eisenberg, 1961; 1964).

$$\xi_3 = 1 / \rho_0 (\phi_2^0 - \phi`_2^0) / (1 - \bar{v}_3 \rho_0) \qquad \text{-------- (6)}$$

where, g is the concentration of component i in grams per grams of water, μ is its chemical potential, ρ_0 is the density of the solvent and T is thermodynamic temperature. ϕ_2^0 and $\phi`_2^0$ are the partial specific volume of the protein at isomolal and isopotential conditions and $\bar{v}_3$ is the partial specific volume of the component 3. The total amount of solvent component (A_3) bound to the protein is related to ξ_3 by the equation (Scatchard, 1946; Stockmayer, 1950).

The same parameter on mol/mol basis $(\delta m_3 / \delta m_2)_{T, \mu_1, \mu_3}$ is also calculated from the following equation

$$(\delta m_3 / \delta m_2)_{T, \mu_1, \mu_3} = M_2 / M_3 \times (\delta g_3 / \delta g_2)_{T, \mu_1, \mu_3} \quad \text{-------- (7)}$$

where, M_2 is the molecular weight of the protein, M_3 is the molecular weight of the cosolvent and $(\delta g_3 / \delta g_2)_{T, \mu_1, \mu_3}$ is the preferential interaction parameter (Timasheff and Kronman, 1959).

RESULTS AND DISCUSSION

CHAPTER – 1

STUDIES ON INVERTASE

1. *Effect of cosolvents on the activity, stability, structure-function, kinetics and preferential interaction studies of invertase*

1. Effect of cosolvents on the activity, stability, structure-function, kinetics and preferential interaction studies of invertase

Increasing the stability of the enzyme against various forms of denaturation is thus a great concern for biotechnological applications for performing enzymatic catalysis at various extreme conditions. Several methods are employed to increase the stability of proteins in operational condition these are chemical modification, use of stabilizing additives, derivatization, immobilization, genetic engineering of enzyme (O'Fagain, 2003; Hassani *et al.,* 2006). Among them, stabilization of enzymes by cosolvents has chosen, these cosolvents added to aqueous solutions of biomolecules profoundly affect protein stability, as well as biochemical equilibria and does not covalently modify the enzyme and very good industrial impact (Klibanov, 1983). Cosolvents are the most prevalent molecule used by nature to protect proteins against loss of activity in various extreme conditions and serve to protect protein against chemical and thermal denaturation (Pradeep and Udgaonkar, 2004; Yancey *et al.,* 1982). Because of the viable and cost factor associated with protein modification, the stability of proteins with cosolvents is recommended.

The stabilizing effect is caused mainly by the hydration in aqueous medium; cosolvents are preferentially excluded from the domain of the protein (Gekko and Morikawa, 1981a). Addition of cosolvents raises the chemical potential of the protein, such a situation is thermodynamically unfavorable and on denaturation the surface of the protein increases (Gekko and Timasheff, 1981a). This increase is accompanied by the exposure of the nonpolar residues buried in the interior of a protein. The resultant increase in nonpolar residues exposed to solvent and hydration of the peptide bond would be translated into enhanced preferential hydration of protein (Xie and Timasheff, 1997a). Petersen *et al.,* (2004) have shown that in case of lysozyme stabilization by sorbitol is due to a solvation of the protein. Different proteins are known to interact with cosolvent molecules in diverse ways and these interactions depend on the physico-chemical properties of the protein.

There are several reports on stabilization of invertase they used different methods such as immobilization ranging from covalent attachement to adsorption or

physical entrapment (Arica and Bayramoglu, 2006; Rebros *et al.,* 2007). Athes and Combes, (1998) have followed the high hydrostatic pressure stability of invertase in presence of polyols. The exact mechanism of enzyme protection by additives is not yet perfectly known and therefore a better understanding of protein-solvent interactions is needed.

This chapter is devoted to understand the effect of selected cosolvents such as glycerol, xylitol and sorbitol on the activity, stability, structure-function relation of invertase at different conditions. The kinetics of invertase at different conditions is followed to understand the mechanism of stabilization. Preferential interaction parameters are calculated to elucidate the mechanism of stabilization by partial specific volume measurements. This is supported by far UV-CD studies, fluorescence spectroscopy and thermal denaturation measurements. Thus, these data would ultimately provide the energetics of the cosolvent-protein interactions to understand at a molecular level the forces that are responsible for the activity and stability of the invertase in these cosolvents.

1. 1. Homogeneity of invertase enzyme

The homogeneity of the invertase was evaluated by SDS-PAGE and isoelectric focusing method by determining its pI. Invertase from *Candida utilis* is a homodimer of two identical subunits with an apparent molecular mass of 150 kDa each and an isoelectric point of 3.35 (Fig. 4A & B) the enzyme activity was carried out at its optimum conditions using sucrose as substrates in pH 4.5 at 55°C.

1. 2. Effect of cosolvents on the activity, stability, structure-function, kinetics and preferential interaction of invertase

The effect of selected cosolvents on the activity of invertase was carried out using different concentration of cosolvents such as glycerol, xylitol, and sorbitol (0-40%). The activity of the enzyme was also checked for its reversibility of its original activity after removal of cosolvents by gel filtration on Sephadex G-25 column or by dialysis. The invertase is activated at low concentration of cosolvents and then gradually decreases with increase in concentration of cosolvents except

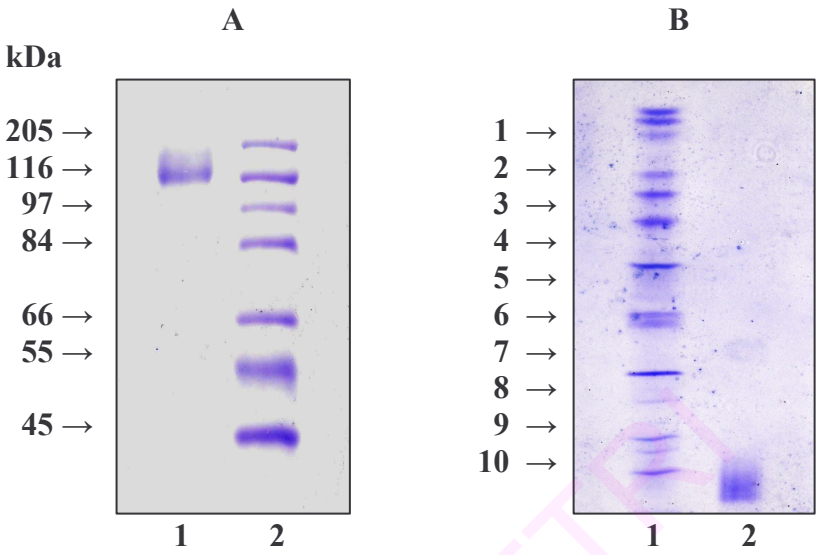

Fig. 4: (A) SDS-PAGE pattern of invertase from *Candida utilis*. (Lane 1) Invertase from *Candida utilis* (150 kDa). (Lane 2) Standard proteins-Myosin (205 kDa), β-Galctosidase (116 kDa), Phosphorylase b (97 kDa), Fructose-6-phosphate kinase (84 kDa), BSA (66 kDa), Glutamine dehydrogenase (55 kDa) and Ovalbumin (45 kDa).

(B) Isoelectric focusing pattern of invertase from *Candida utilis* in Ampholine PAG plate pH 3.5-9.5. (Lane 1) Standard pI marker. (1) Trypsinogen (pI 9.30), (2) Lentil lectin-based band (pI 8.65), (3) Lentil lectin-middle band (pI 8.15), (4) Lentil lectin-acidic band (pI 8.15), (5) Myoglobin-basic band (pI 7.35), (6) Myoglobin-acidic band (pI 6.85), (7) Human carbonic anhydrase B (pI 6.55), (8) Bovine carbonic anhydrase B (pI 5.85), (9) β-lactoglobulin A (pI 5.20) and (10) Soybean trypsin inhibitor (pI 4.55). (Lane 2) Invertase from *Candida utilis* (pI 3.4 ± 0.2).

glycerol. It is clear that the activity of enzyme was enhanced in presence of cosolvents to varying extents in different cosolvents with different concentration (Fig. 5). With increasing concentration of cosolvents such as glycerol, xylitol and sorbitol (0-40%) the enzyme is activated in presence of all the cosolvents and after reaching a peak the enhanced activity gradually decreases with further increase in concentration of cosolvents. In presence of glycerol, the activity of the enzyme was enhanced up to 1.4 fold at 30% concentration. Similarly, in the case of xylitol the enzyme was activated up to the concentration of 30% and above which did not show any activation and it shows a maximum activation of 1.6 fold in 25% xylitol concentration (Table 1). In the case of sorbitol, up to 20% concentration it shows activation above that, it is not showing any activation and a maximum activation of 1.2 fold in 15% sorbitol. These results indicate that the enhancement of activity depends on individual cosolvents.

The enzyme activity was completely regained to its original activity without any fold increase in activity after removal of cosolvents. This result clearly indicates enhancement of invertase activity by cosolvents is completely reversible. Based on the above results the hierarchy of effectiveness of different cosolvents on the activation of invertase is in the order (at 25% concentration)

Xylitol > Glycerol > Sorbitol

In order to understand the mechanism of activation, kinetic experiments were determined in presence of cosolvents as a function of substrate concentration. All the experiments were performed in the same conditions, to compare native and cosolvents treated enzymes. In all the cases catalytic constant (k_{cat}) values are increasing from the control value of 92 to 128, 145 and 113×10^3 min^{-1} with decreased in K_m values from 13 mM to 11, 10 and 12 mM in 30% glycerol, 25% xylitol, and 15% sorbitol concentration respectively leading to higher catalytic efficiency of invertase in presence of cosolvents. The k_{cat}/K_{cat} values also increased from 7×10^3 to 12, 15 and 10×10^3 in presence of above cosolvent concentration respectively. The activation of invertase in presence of cosolvents corresponds to the change in the kinetic parameters leading to a conformational change in native state of enzyme.

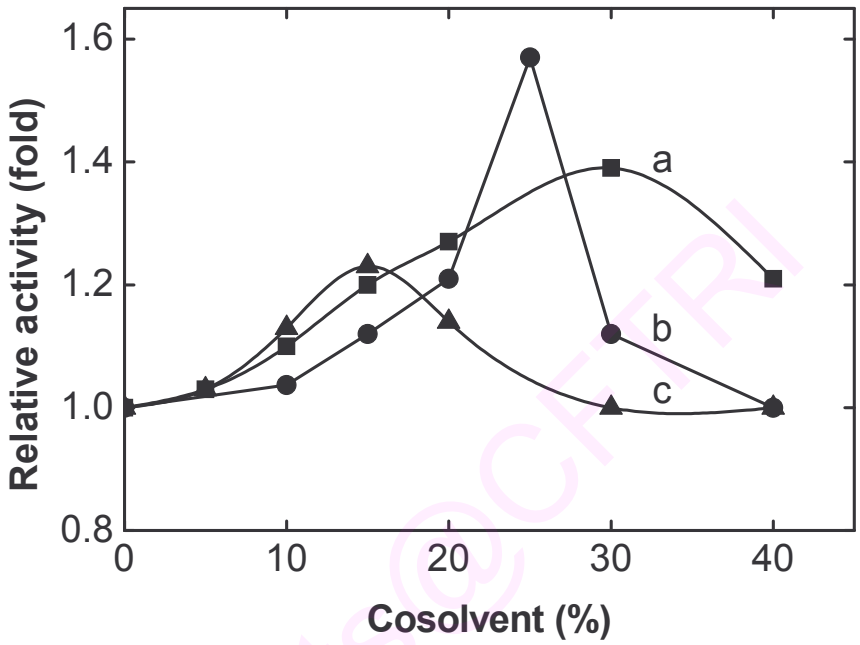

Fig. 5: Relative activity profile of invertase induced by different concentrations of cosolvents in 0.05 M sodium acetate buffer, pH 4.5. The curve shows the change in enzyme activity in the presence of various concentrations of cosolvents (0-40%). (a) in glycerol, (b) in xylitol and (c) in sorbitol.

Table 1: Relative activity of invertase in absence and presence of various concentrations of cosolvents.

Cosolvent concentration (%) (w/v)		Relative activity (fold)*	
		In presence of cosolvent	After removal of cosolvent
Glycerol	0**	1.0	1.0
	5	1.0	1.0
	10	1.1	1.0
	15	1.2	1.0
	20	1.3	1.0
	30	1.4	1.1
Xylitol	0**	1.0	1.0
	10	1.1	1.0
	15	1.1	1.0
	20	1.2	1.0
	25	1.6	1.1
	30	1.1	1.0
Sorbitol	0**	1.0	1.0
	5	1.0	1.0
	10	1.1	1.0
	15	1.2	1.0
	20	1.1	1.0
	30	1.0	1.0

* Relative activity of invertase in 0.05 M sodium acetate buffer, pH 4.5 is 1 fold.

** Controls were run parallely in all these experiments.

The enzymatic function of proteins such as substrate binding, transition state formation and product release require conformational shift from one state to another state, which is influenced by a minor degree of change in surface area. The cosolvents can greatly alter these functions and might affect the association of substrate with enzyme in several ways, through indirect solvation effects (Kim *et al.,* 2003).

The partial specific volume measurements of invertase were carried out with a view to understand the role of water structure around the enzyme molecule, under both isomolal and isopotential conditions. The partial specific volume of invertase was measured as a function of protein concentration in 0.05 M acetate buffer, pH 4.5 at 20°C. The isomolal value was measured after 4 hours of solvent addition, and the isopotential value, which was measured after 36 hours of equilibration in the respective solvents. The values of 0.722 ml/g and 0.723 ml/g were obtained at isomolal and isopotential conditions respectively. A representative plot of cosolvents with invertase is shown in Fig. 6. In all the conditions the apparent partial specific volume was independent of invertase concentration. The apparent partial specific volume of invertase increased as a function of cosolvent concentration at isopotential condition.

The preferential interaction parameters of invertase in cosolvents were calculated using partial specific volume measurements. Preferential interaction parameter for glycerol, xylitol and sorbitol on g/g basis with invertase are shown in Fig. 7. From the Figure it is clear that glycerol has maximum value of preferential interaction parameter. Similar trend was observed in case of xylitol, but to a lesser extent compared to glycerol. However in the case of sorbitol the values increased linearly with increase in the concentration of cosolvents but the increment is lesser compared to both glycerol and xylitol. Partial specific volume, solvent composition, preferential interaction parameter and other related interaction parameters of invertase in glycerol, xylitol and sorbitol are listed in Table 2, 3 and 4 respectively.

The negative interaction value of $(\delta g_3/\delta g_2)_{T,\mu_1,\mu_3}$ in presence of all cosolvents at different concentration was result of preferential exclusion of cosolvents molecules from the protein domain. The preferential exclusion value was maximum in case of

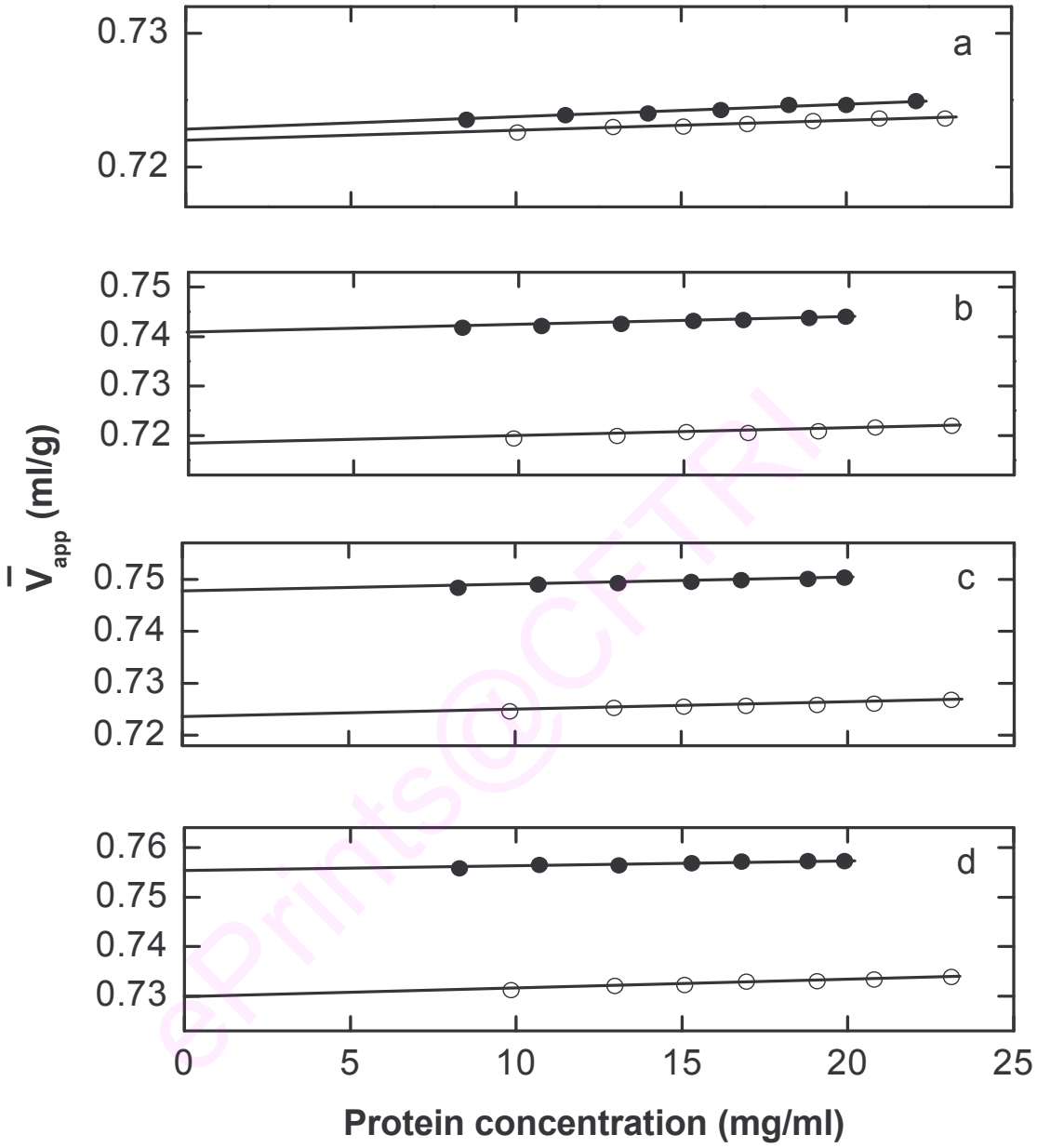

Fig. 6: Apparent partial specific volume of invertase in the presence of different concentrations of cosolvents in 0.05 M sodium acetate buffer, pH 4.5 under isomolal (○) and isopotential (●) conditions at 20°C. (a) control in buffer (0.05 M sodium acetate buffer, pH 4.5), (b) in 40% glycerol, (c) in 40% xylitol and (d) in 40% sorbitol.

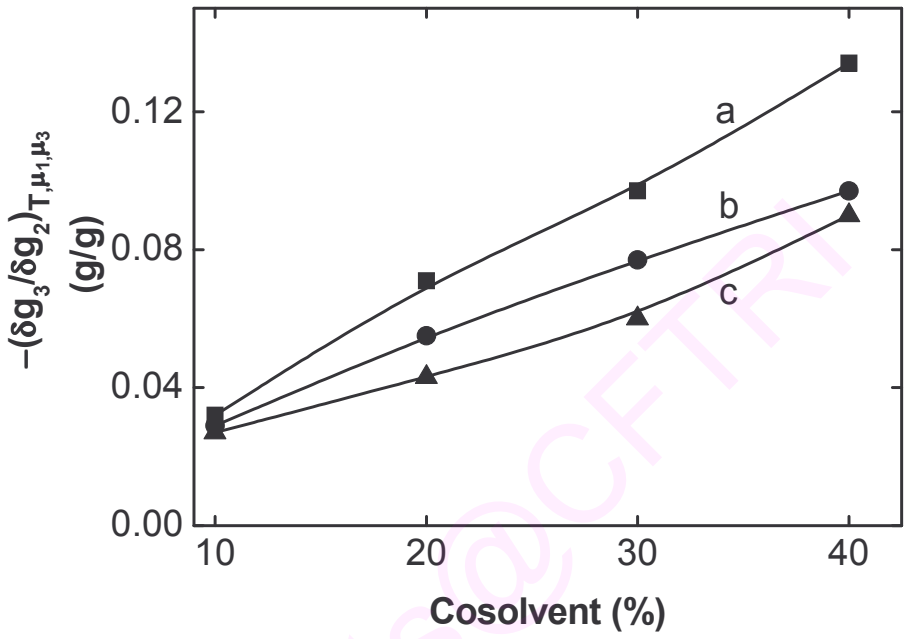

Fig. 7: Effect of cosolvents on the preferential interaction parameter of invertase on g/g basis in the concentration range of 0-40%, w/v. The values were calculated from the isomolal and isopotential partial specific volume measurements. (a) in glycerol, (b) in xylitol and (c) in sorbitol.

Table 2: Apparent partial specific volume and preferential interaction parameter values of invertase as a function of glycerol.

Interaction parameters	Glycerol (%) (w/v)			
	10	**20**	**30**	**40**
ϕ_2^0 (ml/g)	0.720 ± 0.001	0.720 ± 0.001	0.719 ± 0.002	0.719 ± 0.002
$\phi`_2^0$ (ml/g)	0.727 ± 0.001	0.734 ± 0.002	0.737 ± 0.002	0.741 ± 0.002
g_3 (g/g)	0.108	0.236	0.391	0.582
m_3 (mol of solvent per 1000 g H_2O)	1.17	2.57	4.24	6.32
$(\delta g_3/\delta g_2)T,\mu_1,\mu_3$ (g/g)	−0.032 ± 0.008	−0.071 ± 0.012	−0.097 ± 0.015	−0.134 ± 0.029
$(\delta g_1/\delta g_2)T,\mu_1,\mu_3$ (g/g)	0.293 ± 0.022	0.298 ± 0.038	0.249 ± 0.030	0.230 ± 0.028
$(\delta m_3/\delta m_2)T,\mu_1,\mu_3$ (mol/mol)	−89.8 ± 7.3	−199.3 ± 36.5	−275.19 ± 39.2	−378.9 ± 42.5

Table 3: Apparent partial specific volume and preferential interaction parameter values of invertase as a function of xylitol.

Interaction parameters	Xylitol (%) (w/v)			
	10	20	30	40
ϕ_2^0 (ml/g)	0.722 ± 0.001	0.724 ± 0.002	0.725 ± 0.002	0.724 ± 0.002
$\phi`_2^0$ (ml/g)	0.731 ± 0.002	0.740 ± 0.002	0.746 ± 0.002	0.748 ± 0.002
g_3 (g/g)	0.107	0.232	0.378	0.554
m_3 (mol of solvent per 1000 g H_2O)	0.71	1.52	2.48	3.64
$(\delta g_3/\delta g_2)T,\mu_1,\mu_3$ (g/g)	−0.029 ± 0.006	−0.055 ± 0.010	−0.077 ± 0.015	−0.097 ± 0.020
$(\delta g_1/\delta g_2)T,\mu_1,\mu_3$ (g/g)	0.274 ± 0.065	0.237 ± 0.054	0.203 ± 0.045	0.175 ± 0.035
$(\delta m_3/\delta m_2)T,\mu_1,\mu_3$ (mol/mol)	−50.25 ± 6.05	−94.06 ± 9.43	−131.19 ± 10.2	−166.18 ± 14.9

Table 4: Apparent partial specific volume and preferential interaction parameter values of invertase as a function of sorbitol.

Interaction parameters	Sorbitol (%) (w/v)			
	10	**20**	**30**	**40**
ϕ_2^0 (ml/g)	0.719 ± 0.001	0.727 ± 0.002	0.729 ± 0.002	0.730 ± 0.002
$\phi`_2^0$ (ml/g)	0.728 ± 0.002	0.741 ± 0.002	0.747 ± 0.002	0.755 ± 0.003
g_3 (g/g)	0.107	0.230	0.374	0.546
m_3 (mol of solvent per 1000 g H_2O)	0.58	1.26	2.05	2.99
$(\delta g_3/\delta g_2)T,\mu_1,\mu_3$ (g/g)	−0.027 ± 0.004	−0.043 ± 0.008	−0.060 ± 0.009	−0.090 ± 0.013
$(\delta g_1/\delta g_2)T,\mu_1,\mu_3$ (g/g)	0.249 ± 0.060	0.189 ± 0.032	0.161 ± 0.020	0.165 ± 0.032
$(\delta m_3/\delta m_2)T,\mu_1,\mu_3$ (mol/mol)	−38.01 ± 5.2	−61.96 ± 8.2	−86.00 ± 9.5	−128.00 ± 22.4

40% glycerol with a value of −0.134 g/g. This exclusion, in turn raises the chemical potential of the protein by slightly decreasing the surface tension of water which is thermodynamically an unfavorable reaction due to general and non-specific interaction of cosolvents molecules which are stastically distributed over the entire protein solvent surface (Gekko and Timasheff, 1981a).

The partial specific volume results indicate that the enzyme is stabilized in all cosolvents systems used in a concentration dependent manner and the preferential interaction parameter indicates the extent of polyols exclusion from the protein domain. The maximum hydration was observed in case of 40% glycerol where the preferential interaction parameter was −0.134 g/g and the lowest of −0.027 g/g in 10% sorbitol. The same interaction on a mol/mol basis was also calculated, showing highest value of −379 in the case of 40% glycerol and the lowest of −38 in 10% sorbitol. The value of $(\delta g_1/\delta g_2)_{T,\mu_1,\mu_3}$ are shown in Table 2 to 4, which as a value of 0.298 g/g, being highest in case of 20% glycerol and the lowest value of 0.161 g/g in 30% sorbitol.

Far UV-CD and fluorescence studies have been used to prove the conformational changes in invertase in different cosolvents. Far UV-CD spectra of native invertase exhibit only one minima at 218 nm indicating beta sheet rich structure (Fig. 8). In the presence of 30% glycerol, 20% sorbitol and 25% xylitol an increase in ellipticity values in the region of 210-220 nm was observed. There was minor significant change in the structural features of invertase in presence of cosolvents at optimum condition with slight increase in α-helical content from 4% to 6%, 8%, 5% and slight changes in β-structure from 70% in control to 67%, 66% and 66% in presence of 20% sorbitol, 25% xylitol and 30% glycerol concentration respectively. Slight changes in the aperiodic structure from 26% to 27%, 26% and 29% in presence of above cosolvent concentrations was observed. These structural studies indicate that the cosolvents have minor affect on the secondary structural characteristic of invertase at optimum conditions. Fluorescence emission spectra of invertase as a function of cosolvent concentrations are shown in Fig. 9A-C. At optimum conditions the intrinsic fluorescence spectra of invertase in presence of

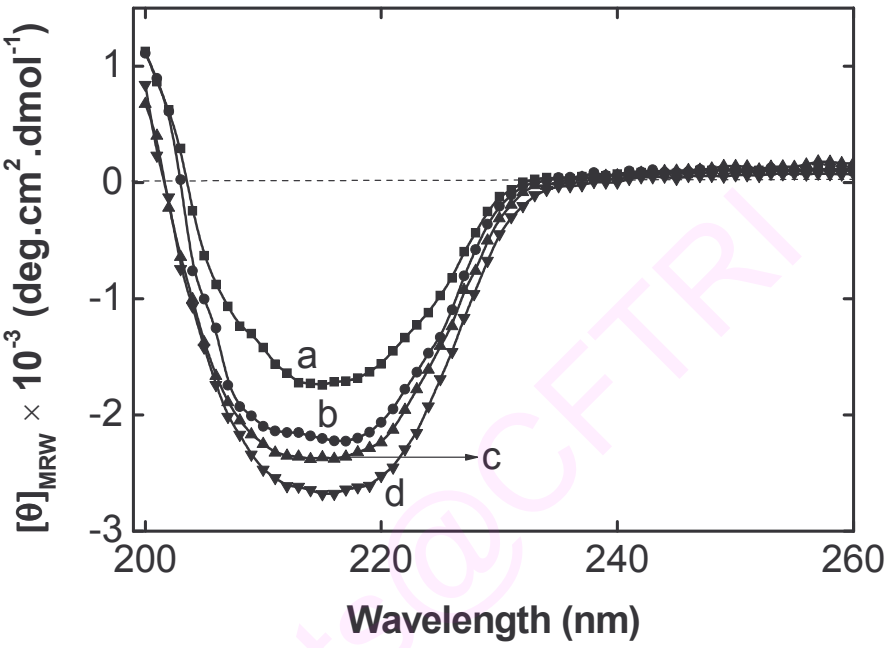

Fig. 8: Far UV-CD spectra of invertase in the presence of different concentrations of cosolvents in 0.05 M sodium acetate buffer, pH 4.5 at 25°C. (a) control in buffer (0.05 M sodium acetate buffer, pH 4.5), (b) in 20% sorbitol, (c) in 30% glycerol and (d) in 25% xylitol.

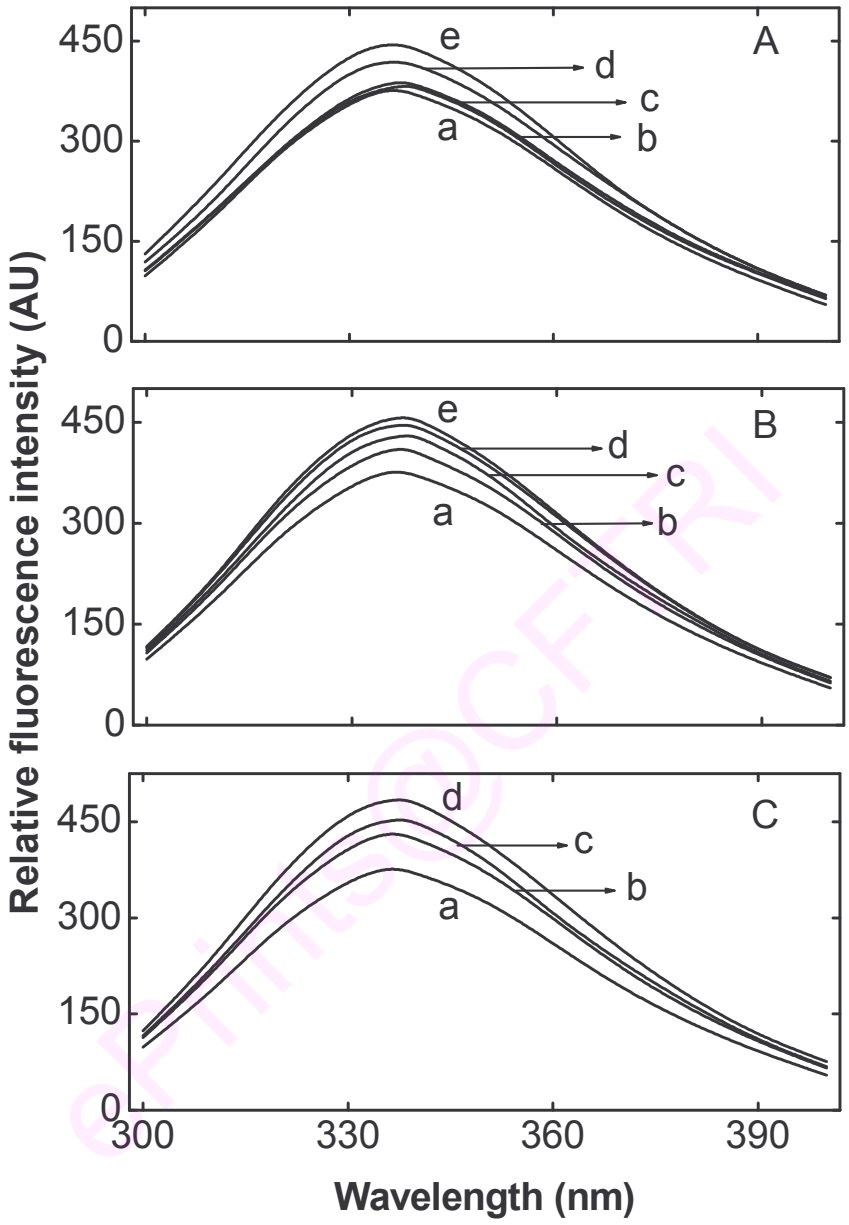

Fig. 9: Intrinsic fluorescence emission spectra of invertase in the presence of different concentrations of cosolvents in 0.05 M sodium acetate buffer, pH 4.5. Emission spectra recorded over a range of 300-400 nm. (a) control in buffer (0.05 M sodium acetate buffer, pH 4.5), (b) in 10%, (c) in 20%, (d) in 30% and (e) in 40% cosolvents. (A) in glycerol, (B) in xylitol and (C) in sorbitol.

cosolvents did not show any influence on both fluorescence emission intensity and emission maximum except minor increase in fluorescence emission intensity.

The increase in the activity at optimum condition has been related to the preferential hydration of proteins (Gekko and Morikowa, 1981b; Gekko and Timasheff, 1981b). From the structural and preferential interaction studies suggest that the cosolvents stabilize the invertase by preferential hydration mechanism. This might be changing the water structure around the active site or enzyme as a whole could lead to functionally more active state in presence of cosolvents. These cosolvents exhibit greater protective effects in a phenomenon that possibly involves their ability to alter the viscosity and water activity of the medium with or without bringing conformational changes in the enzyme.

A number of studies by Timasheff and co-workers on different cosolvent system such as salts (Arakawa and Timasheff, 1982b), amino acids (Arakawa and Timasheff, 1983), polyhydric alcohols (Gekko and Morikawa, 1981a; Gekko and Timasheff, 1981b) and sugars (Lee and Timasheff, 1981) with a variety of protein as explained the phenomenon of preferential hydration. This hydration layer around the protein cannot be penetrated by cosolvent molecule because of the strong force of attraction between water molecule and the protein surface.

1. 3. Effect of cosolvents on the activity, stability, structure-function and kinetics of invertase at higher temperature

Temperature is the most important factor affecting protein stability and functionality. There is no general mechanism to describe the effect of temperature on the structure and function of proteins due to its complicated effects. In general, the higher the temperatures lower the protein stability. Cosolvents are very good stabilizing agents and have been used for stabilization of variety of different proteins (Gekko and Timasheff, 1981a; Xie and Timasheff, 1997a; Kaushik and Bhat, 1998; Petersen *et al.,* 2004). Extensive studies have shown that the effect of different polyols on the thermal stability of papain have demonstrated that stabilizing effect of polyols is correlated with the preferential interaction parameter at different concentrations (Sathish *et al.,* 2007).

This leads to the preferential hydration of proteins as observed by other workers. It is important to know, whether addition of cosolvents to invertase at higher temperature brings about any change in the kinetics and structure, therefore the kinetics and thermodynamic properties of enzymes in presence of different cosolvents were carried out.

The effect of selected cosolvents on the thermal stability of invertase was measured through activity, structural and thermal transition studies. An increase in temperature beyond optimum caused a rapid inactivation of the enzyme with 90% loss in activity was observed at 80°C. The enzyme activity was measured after heating the enzyme at 80°C for 20 min in presence and absence of cosolvents. The results of residual specific activity versus concentration of cosolvents in (%) are shown in Fig. 10. All the cosolvents used (0-40% concentration) were able to protect the activity against heat inactivation. The native enzyme looses nearly 90% of its activity and in presence of cosolvents the enzyme retained maximum of 80% of its activity in presence of 40% xylitol. In case of 40% glycerol the enzyme retained 70% of its activity and compared to glycerol and xylitol, sorbitol shows to be less effective it retains only 45% of its residual activity at 40% concentration (Table 5).

All the results indicated increased thermal stability of invertase in presence of cosolvents. From the Table 5 it is clear that xylitol and glycerol at 40% concentration offer maximum protection against thermal inactivation as evidenced by maximum recovery of residual specific activity values of nearly 20 and 15% respectively after removing the cosolvents by gel filtration on Sephadex G-25 column. Sorbitol found to be least effective with a recovery value of only 13% at 40% sorbitol concentration. From the kinetic analysis, the data shows decrease in K_m values from 34 mM in the absence of cosolvents to 18, 17 and 21 mM in presence of 40% glycerol, xylitol and sorbitol with increase in catalytic constant (k_{cat}) values from 9 to 65, 74 and 41×10^3 min^{-1} and k_{cat}/K_m values also increased from 0.3×10^3 to 3.6×10^3, 4.4×10^3, and 1.9×10^3 in the presence of 40% glycerol, xylitol and sorbitol concentration respectively. The protection of activity in presence of cosolvents corresponds to the change in the kinetic parameters leading to a conformational change in cosolvent treated enzymes.

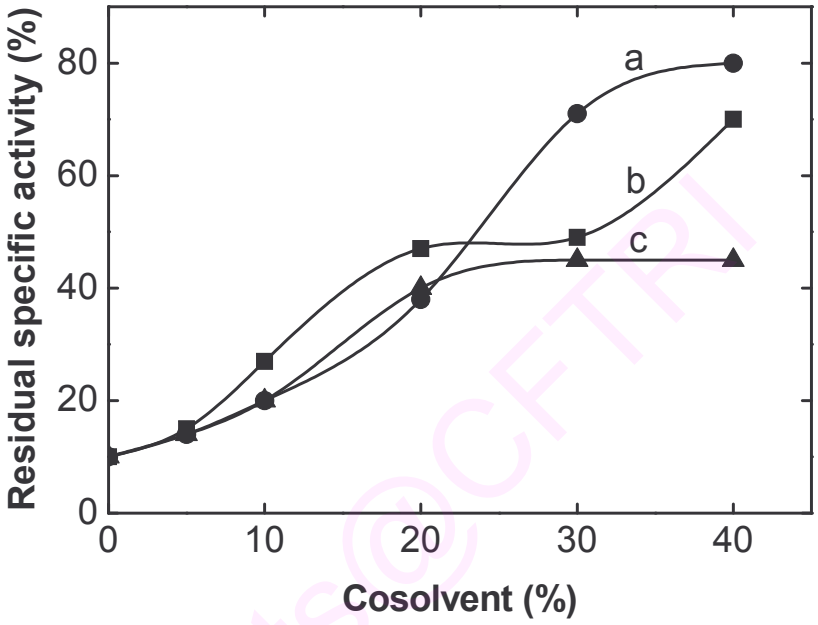

Fig. 10: Thermal inactivation profile of invertase in the presence of different concentrations of cosolvents in 0.05 M sodium acetate buffer, pH 4.5. The reaction mixture was exposed to 80°C for 20 min with and without cosolvents (0-40%). (a) in xylitol, (b) in glycerol and (c) in sorbitol.

Table 5: Residual specific activity of invertase as a function of cosolvent concentrations after exposure to 80°C for 20 min.

Cosolvent concentration (%) (w/v)		Residual specific activity (%)*	
		In presence of cosolvent	After removal of cosolvent
Glycerol	0**	10 ± 1	2 ± 0.2
	5	15 ± 1	3 ± 0.3
	10	27 ± 2	4 ± 0.5
	20	47 ± 2	10 ± 3
	30	49 ± 2	9 ± 1
	40	70 ± 3	15 ± 1
Xylitol	0**	10 ± 1	2 ± 0.2
	5	14 ± 1	6 ± 0.5
	10	20 ± 2	8 ± 0.9
	20	38 ± 2	10 ± 1
	30	71 ± 3	15 ± 1
	40	80 ± 4	20 ± 2
Sorbitol	0**	10 ± 1	2 ± 0.2
	5	14 ± 1	4 ± 0.3
	10	20 ± 2	6 ± 0.5
	20	40 ± 2	7 ± 0.5
	30	45 ± 3	13 ± 1
	40	45 ± 3	13 ± 1

* Heat treated invertase (80°C for 20 min) in 0.05 M sodium acetate buffer, pH 4.5.

** Controls were run parallely in all these experiments.

Secondary structural studies of invertase were carried out after heat treatment at 80°C for 20 min in presence and absence of cosolvents (Fig. 11). From the secondary structural analysis at higher temperature there was no significant change in the secondary structural content of invertase with addition of cosolvents. The α-helix content increased from 6% in control to 7%, 8% and 7% with slight changes in β-structure from 57% in control to 61%, 62% and 61% in presence of 20% sorbitol, 25% xylitol and 30% glycerol respectively. Fluorescence spectra of invertase have taken after invertase was exposed to 80°C for 20 min with and without cosolvents (Fig. 12A-C). From the spectra at higher temperature there was a decrease in fluorescence intensity with shift in emission maximum, but in presence of cosolvents both increase in fluorescence intensity up to 50% with blue shift in the emission maximum was observed.

The stabilization of invertase as evident by enzyme activity measurements has been further confirmed by the measurement of thermal denaturation temperature (T_m) of invertase both in presence and absence of cosolvents (Fig. 13A-C). The changes in the absorbance of protein at 287 nm were monitored as a function of temperature in the range of 30-95°C. The thermal denaturation curve analysis of invertase indicated a shift in the apparent thermal denaturation temperature as a function of different cosolvent concentrations. The apparent T_m shifted from a control value of 75°C to a maximum of 85°C in presence of 30% glycerol. The apparent T_m rose to 79, 82 and 84°C in presence of 10, 20 and 30% xylitol respectively. In case of 10, 20 and 30% sorbitol and glycerol the T_m rose to 77, 79 and 81°C in sorbitol and 79, 82 and 85°C in glycerol respectively.

All these results suggest that these cosolvents exert a stabilizing effect on invertase at higher temperature. Cosolvents were found to protect the enzymes against thermal inactivation. The protection was a time and dose dependent phenomenon. The insertion of a protein in unusual environments induces a variety of conformational responses as an adaptation to the new perturbing influence. The macromolecule strains back to cope with the new stress. The protein structural architecture shows a great deal of flexibility on responding to the different environmental changes, shifting

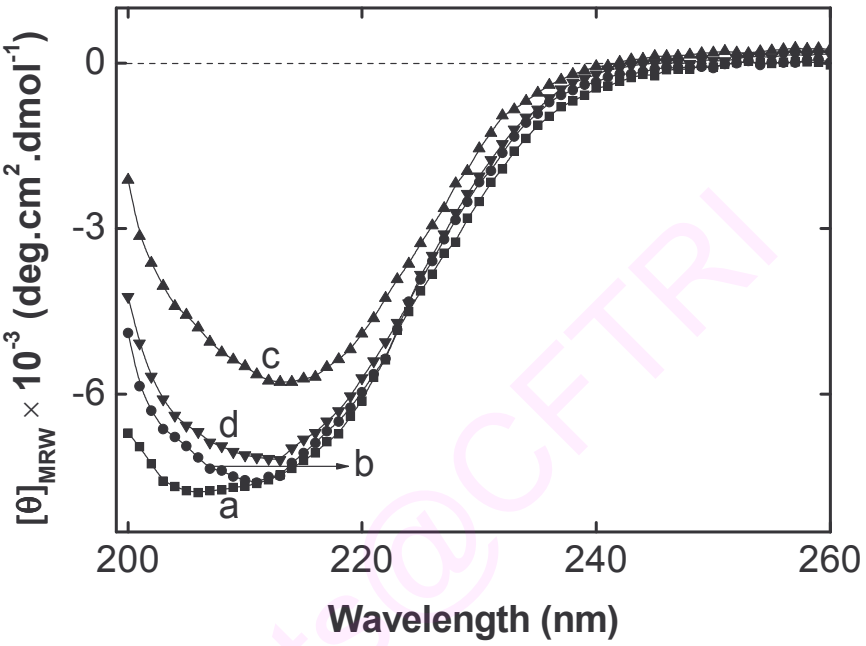

Fig. 11: Far UV-CD spectra of invertase in the presence of different concentrations of cosolvents in 0.05 M sodium acetate buffer, pH 4.5 after exposure to 80°C for 20 min. (a) control in buffer (0.05 M sodium acetate buffer, pH 4.5), (b) in 30% glycerol, (c) in 30% xylitol and (c) in 30% sorbitol.

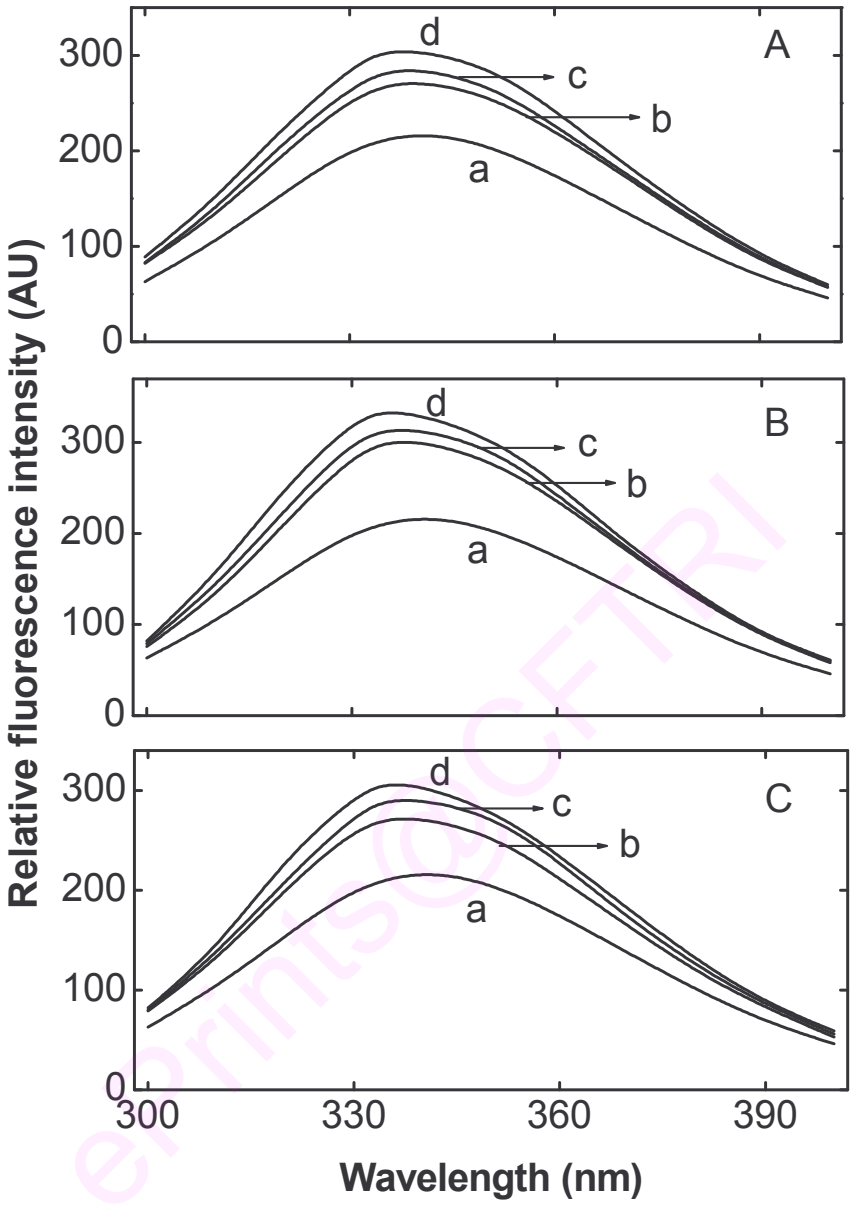

Fig. 12: Intrinsic fluorescence emission spectra of invertase in the presence of different concentrations of cosolvents in 0.05 M sodium acetate buffer, pH 4.5 after exposure to 80°C for 20 min. Emission spectra recorded over a range of 300-400 nm. (a) control in buffer (0.05 M sodium acetate buffer, pH 4.5), (b) in 10%, (c) in 20% and (d) in 30% cosolvents. (A) in glycerol, (B) in xylitol and (C) in sorbitol.

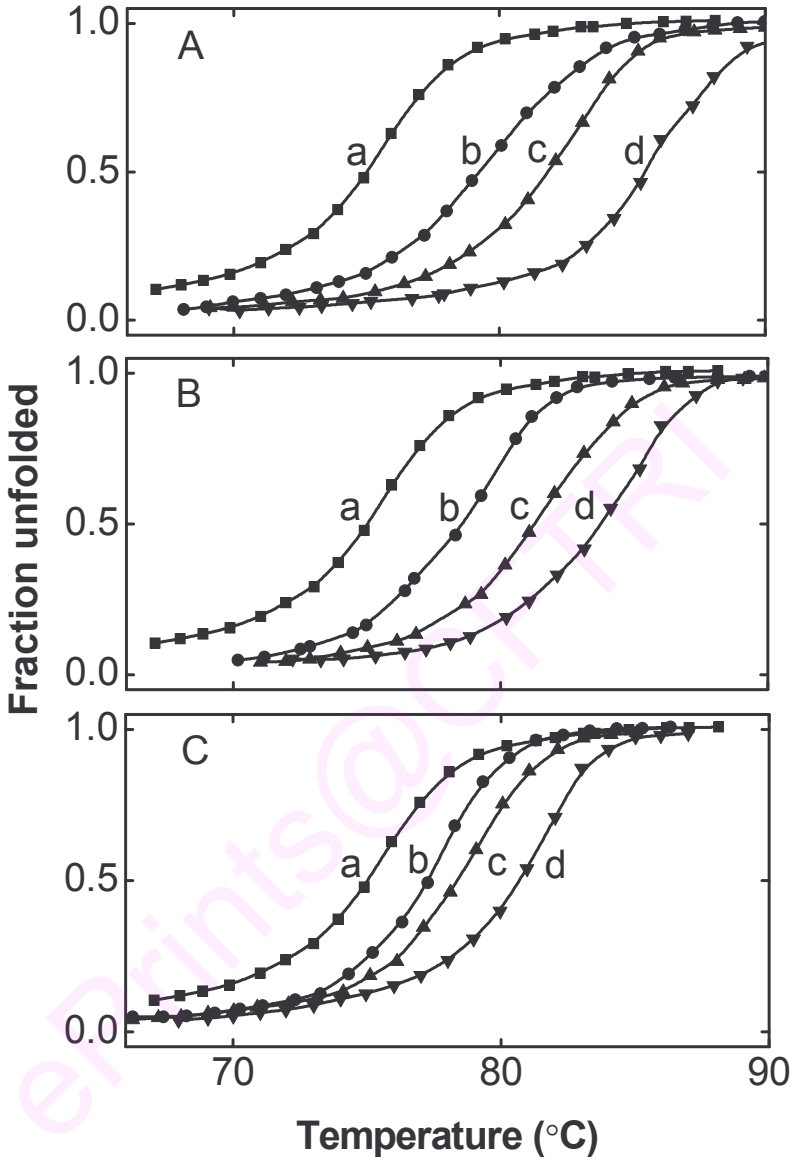

Fig. 13: Apparent thermal denaturation curves of invertase in the presence of different concentrations of cosolvents in 0.05 M sodium acetate buffer, pH 4.5. The absorption spectra were recorded as a function of temperature at 287 nm. (a) control in buffer (0.05 M sodium acetate buffer, pH 4.5), (b) in 10%, (c) in 20% and (d) in 30% cosolvents. (A) in glycerol, (B) in xylitol and (C) in sorbitol.

the thermodynamic behavior along the chemical coordinates of the protein-solvent integrated system.

1. 4. Stability of invertase in presence of cosolvents under alkaline condition

The pH of the protein solution plays a major role in determining the 3-D structure of proteins or enzymes. pH can have an effect of the state of ionization of acidic or basic amino acids. An alteration in pH, either in acidic or alkaline region affects the ionization of ionizable amino acid residues and subsequently affects the structural integrity of the protein. This can lead to altered protein conformation and enzyme may be inactivated. Changes in pH may not only affect the shape of an enzyme but it may also change the shape or charge properties of the substrate so that either the substrate cannot bind to the active site or it cannot undergo catalysis. Invertase displayed high activity over a broad pH range (4-7) with optimum activity at pH 4.5 (Belcarz *et al.,* 2002; Chavez *et al.,* 1997).

Invertase displayed very high activity over a pH range (4-7) with optimum activity at pH 4.5 (Belcarz *et al.,* 2002). The range of pH stability of invertase was found to be 4-7 (Chavez *et al.,* 1997). An increase in pH beyond optimum caused a rapid inactivation of the enzyme and 94% loss in activity was observed at pH 9.0. It is reported that the invertase isolated from different yeast and fungi, the optimum range of pH was found to be 3.8 to 5.5 (Akgol *et al.,* 2001; Hocine *et al.,* 2000). pH can have, an effect of the state of ionization of acidic or basic amino acids. This can lead to altered protein recognition or an enzyme which might become inactive. The pH stability of invertase at alkaline pH (9.0) was measured with cosolvents, native enzyme loses 94% of the residual specific activity. Glycerol has not shown any protecting effect only xylitol and sorbitol shown protection against pH denaturation (Fig. 14). In case of xylitol 68% of the activity is retained at 40% concentration and sorbitol show 48% retention at 40% respectively (Table 6).

At alkaline condition kinetic parameters were determined, Michaelis (K_m) and catalytic constant (k_{cat}) of invertase shows a variable changes in presence of cosolvents at alkaline pH. In the absence of cosolvents at alkaline pH the apparent K_m

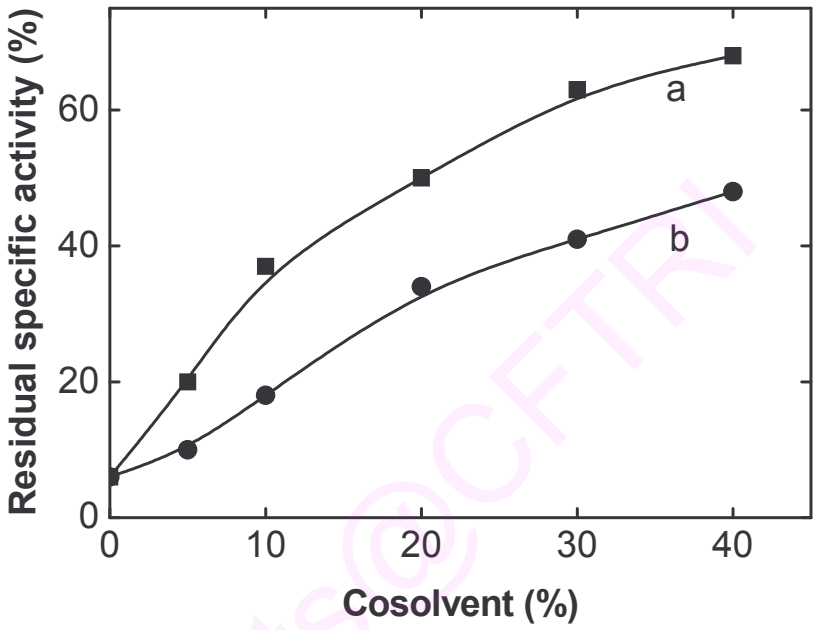

Fig. 14: pH inactivation profile of invertase in the presence of different concentrations of cosolvents in 0.05 M Tris-HCl buffer, pH 9.0. (a) in xylitol and (b) in sorbitol.

Table 6: Residual specific activity of invertase as a function of cosolvent concentrations at pH 9.0.

Cosolvent concentration (%) (w/v)		Residual specific activity (%)*	
		In presence of cosolvent	After removal of cosolvent
Xylitol	0**	6 ± 0.5	0
	5	20 ± 2	3 ± 0.3
	10	37 ± 2	5 ± 0.4
	20	50 ± 3	10 ± 1
	30	63 ± 3	16 ± 2
	40	68 ± 3	20 ± 2
Sorbitol	0**	6 ± 0.5	0
	5	10 ± 1	2 ± 0.2
	10	18 ± 2	5 ± 0.4
	20	34 ± 2	7 ± 1
	30	41 ± 2	8 ± 1
	40	48 ± 2	10 ± 1

* Invertase in 0.05 M Tris-HCl buffer, pH 9.0.

** Controls were run parallelly in all these experiments.

for invertase was 29 mM, and the catalytic constant was 5.6×10^3 min^{-1}. In the presence of 40% sorbitol the K_m was 22 mM and k_{cat} was 44×10^3 min^{-1} where as in the presence of 40% xylitol the K_m droped to 19 mM and the k_{cat} rose to 63×10^3 min^{-1}. The k_{cat}/K_m value increases from 0.2×10^3 in control to 2×10^3 and 3.3×10^3 with above cosolvents concentration. The decrease in K_m and increase in the catalytic constant suggest that invertase is achieving a maximal catalytic efficiency in presence of cosolvents. The far UV-CD spectra of invertase in presence of cosolvents at pH 9.0 are shown in Fig. 15. At alkaline pH the secondary structural content of invertase were not affected significantly in presence of cosolvents. There were no significant changes in the secondary structural content of invertase in presence of polyols at alkaline pH, except minor increase in α-helical content from 3% in control to 6% and 7% with slight decrease in β-sheet content from 65% to 64% and 63% in presence of 30% sorbitol and xylitol respectively.

The fluorescence spectra of invertase in presence of xylitol and sorbitol at pH 9.0 are shown in Fig. 16A & B. There was a decrease in emission intensity in presence of higher pH but in presence of cosolvents there was a slight increase in emission intensity with blue shift in the emission maximum. The increase in the intensity is gradual over the entire range of cosolvent concentrations. The wavelength of maximum emission shifted from a control value of 342 nm at alkaline pH to a value of 336 nm in presence of cosolvents.

The above results clearly suggest that all the cosolvents increase the activity, thermal stability and pH stability of the enzyme in a concentration dependent manner. There have been several ideas as to how these cosolvents exert their effects on protein stability and this has given rise to a number of concepts (Bolen and Baskakov, 2001; Gekko and Timasheff, 1981a; Lin and Timasheff, 1996; Schellman, 2003; Street *et al.,* 2006; Timasheff, 2002a; Tiwari and Bhat, 2006). In turn, the mechanism by which individual cosolvents bring about the stability may be different depending upon the nature of the cosolvents used.

These cosolvents exhibit greater protective effects in a phenomenon that possibly involves their ability to alter the viscosity and water activity of the medium

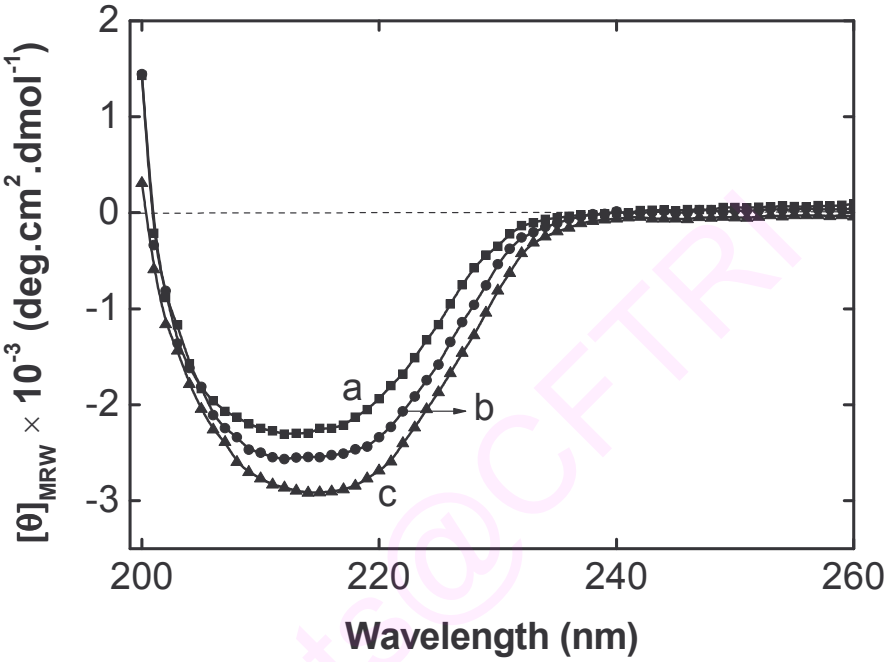

Fig. 15: Far UV-CD spectra of invertase in the presence of different concentrations of cosolvents in 0.05 M Tris-HCl buffer, pH 9.0 at 25°C. (a) control in buffer (0.05 M Tris-HCl buffer, pH 9.0), (b) in 30% sorbitol and (c) in 30% xylitol.

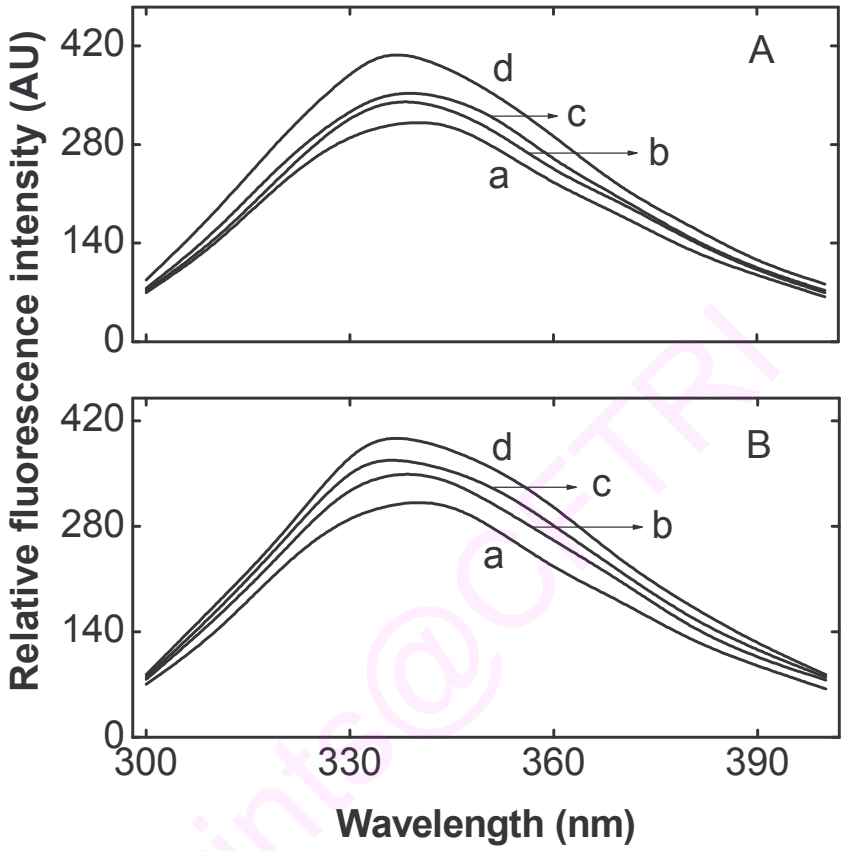

Fig. 16: Intrinsic fluorescence emission spectra of invertase in the presence of different concentrations of cosolvents in 0.05 M Tris-HCl buffer, pH 9.0. Emission spectra recorded over a range of 300-400 nm. (a) control in buffer (0.05 M Tris-HCl buffer, pH 9.0), (b) in 10%, (c) in 20% and (d) in 30% cosolvents. (A) in xylitol and (B) in sorbitol.

without any conformational changes in the enzyme. Further evidence of cosolvent affecting conformational changes in native state comes from the modulation of the enzymatic function by osmolytes (Burg *et al.,* 1999; Fields *et al.,* 2001).

Far UV-CD spectrum of the protein arises primarily from the spatial arrangements of amide groups, a small increase in molar ellipticity implies that the native state ensemble is shifted to favor species with enhanced order of secondary structure (Sreerama and Woody, 1993), these data supported by fluorescence spectra by moderate enhancement in emission intensity making protein more hydrophobic. It has also been reported earlier that compatible osmolytes might be expected to affect the association of substrate with enzyme in several ways, through solvation effects on substrate or enzyme active site. The results suggest that both kinetic and catalytic constant of invertase are slightly perturbed by the cosolvents used.

The stabilization effect of proteins by cosolvents at higher temperature and at different pH depends on the physicochemical properties of the protein, and also known to be highly pH dependent (Kaushik and Bhat, 1998; Xie and Timasheff, 1997c). Haque *et al.,* (2005), showed that stabilizing effect of lysozyme and ribonuclease A, increases with decreasing the pH and cosolvents have been shown to stabilize the acid unfolded state of cytochrome C, by strengthening hydrophobic interactions by overcoming electrostatic repulsions among the charged residues at low pH (Kamiyama *et al.,* 1999). Cosolvents have been earlier known to increase the thermal transition temperature in different pH. Petersen *et al.* (2004), conducted a series of experiments at different pH values and showed that sorbitol was excellent stabilizing agent in all condition both at acidic and alkaline pH with a remarkable protection up to 13°C in the pH range of 9.5-10.5 and showed that this may be associated with a change in the local dielectric constant of the protein surface.

However, from thermal, fluorescence, structural and kinetic studies of invertase with cosolvents it has been observed that increase in preferential hydration, correlated with increase in surface tension of water in their presence. Among cosolvents, xylitol stabilizes the activity of invertase when incubated at higher temperature to the maximum extent with change in the kinetic parameter compared to

the native sample. From the kinetic studies using staphylococcal nuclease by xylose (Frye and Royer, 1997), showed that xylose stabilize the protein by surface tension effect. From the fluorescence studies of invertase in presence of these cosolvents it is clear that change in the microenvironment of aromatic chromophore of invertase is occurring to different extents and altering in the dielectric constant around the microenvironment of tryptophan residues (Petersen *et al.,* 2004). In a defined system one can use fluorescence, especially in the presence of cosolvents only to get an indication of the changing tryptophan microenvironment and must be interpreted with great caution. Therefore, the blue shift observed in the λ_{max} in presence of cosolvents at higher temperature indicated the increased apolar microenvironment around tryptophan. In another study, using fluorescence anisotropy, it has been shown that sorbitol can reduce the internal motions in the nucleocapsid protein of rhabdovirus and favors a more compact state of the protein (Majumder *et al.,* 2001).

Thus, cosolvents known to strengthen the hydrophobic interaction and increasing the thermal denaturation temperature of protein compared to native protein. From the thermal stability experiments of β-lactoglobulin in the presence of aqueous solution of alcohols and polyols (Romero *et al.,* 2007), by fluorescence measurements and their thermodynamic results show that alcohol denaturating effect diminishes gradually with their increasing number of OH group. The increased thermal stability of several proteins in many polyols is interpreted as increase in surface tension of water in presence of these cosolvents thus leading to the preferential hydration in these cosolvents (Kaushik and Bhat, 1998). Xia *et al.,* (2007) have reported protective effects of osmolytes such as sucrose and glycerol on arginine kinase and their results suggest that these osmolytes prevented inactivation of the enzyme by increasing the transition free energy changes.

Stabilizing solutes (sugars and polyols) have been found to preferentially excluded from contact with the surface of the protein, and the protein is said to preferentially hydrated. Preferential exclusion, in a thermodynamic sense, means the solute (ligand) has negative binding to the protein. The degree of exclusion is greater for the denatured than for the native state because unfolding leads to a greater surface area of contact between the protein and the solvent. Thus even though there is an increase in the free energy of the native state, there is a greater increase in the free

energy of the denatured state. The result is an increase in the stability of the native state by mechanism as explained by Gekko and Morikawa, (1981b), Gekko and Timasheff, (1981a) and Xie and Timasheff, (1997b).

The cohesive effect is reflected as surface tension increase as more sugar is added (Lee and Timasheff, 1981). Increase in surface tension result in the depletion of solute at the air-solution interface. It follows that, if a solute increase the surface tension of water, more work is required to increase the surface area of the cavity occupied by the protein in solution (Arakawa and Timasheff, 1982a; Kaushik and Bhat, 1998; Lee and Timasheff, 1981). The preferential hydration effect should lead to a loss in the entropy of the solvation upon protein denaturation, rendering the unfolded state even more unstable, and resulting in a shift of the equilibrium in favor of the native state (Kaushik and Bhat, 2003).

The present study as seen from the activity, kinetics and structural measurements of the invertase at optimum, high temperature and alkaline pH conditions, cosolvents were protecting the invertase under all conditions. The results presented here in conjunction with those obtained for several other proteins in the literature. Bulk solution physicochemical properties of cosolvents reflecting structure of aqueous solutions are important contributors toward the stabilizing effect of proteins, in addition to the peptide group contribution, much would also depend on the nature of the surface groups in proteins, which may either repel the solute molecules or have favorable interactions with them. A balance of all these forces would contribute toward the overall stabilizing effect of enzymes by these cosolvents. From thermal denaturation, fluorescence and secondary structural studies of invertase in presence of different cosolvents indicate that cosolvents have stabilizing affect against thermal denaturation. The result provides a strategy to improve both activity and stability of invertase by cosolvent addition. An extensive study is required to generate extremely active and stable invertase that is especially desirable in industry. Such study will enable us to understand the catalytic mechanism, thermal stability, and pH stability of invertase.

CHAPTER – 2

STUDIES ON NUCLEASE P1

2a. Effect of cosolvents on the activity, stability, structure-function and kinetic studies of nuclease P1

2a. Effect of cosolvents on the activity, stability, structure-function and kinetic studies of nuclease P1

Cosolvents play a crucial role in protein stability; they can induce unfolding or folding, and may as well lead to significant changes of the critical parameters, i.e. the critical temperature and the critical pressure which determine the phase boundaries between native and denatured state (Robinson and Cho, 1999). Cosolvent stabilizes protein, not interacting with them directly but by altering the solvent properties of the surrounding water (Timasheff, 1993). These solutes are amino acids and derivatives, polyols and sugars, urea, GuHCl. Except for urea and GuHCl they are often called compatible solutes based on the hypothesis that these solutes do not interact with macromolecules in detrimental ways (Yancey, 2005). Cosolvents have been used as protein stabilizers for many years, but mechanism of stabilization effect was not clear.

Timasheff and co-workers demonstrated that sugars and other polyhydric alcohols are preferentially excluded from the surface of the protein. Thus, the folded state of the protein is stabilized relative to the unfolded state because it exposes less surface area from which the cosolvent must be excluded. Thus, the relative stabilization does not involve contact with the cosolvent. Rather, the hydration layer around the protein is altered (Arakawa and Timasheff, 1985; Gekko and Timasheff, 1981b; Lin and Timasheff, 1996; Xie and Timasheff, 1997a and b).

Nuclease P1 is found in the mold *Penicillium citrinum*. It belongs to a class of mostly secreted, extracellular endonucleases which cleaves single-stranded RNA and DNA into 5'-mononucleotides (Volbeda *et al.,* 2004). They exhibit high selectivity for single-stranded nucleic acids and single-stranded regions in double-stranded nucleic acids and hence they are widely used as probes for the structural determination of nucleic acids, mapping mutations and studying the interactions of DNA with various intercalating agents. Intracellularly, some of them have been implicated in recombination, repair and replication (Volbeda *et al.,* 2004). Nuclease P1 may be inactivated by slight changes in the physicochemical parameters such as temperature, pressure, pH and ionic strength.

In this chapter, effect of selected cosolvents such as glycerol, sucrose and trehalose on the activity and stability of nuclease P1 in different conditions such as thermal and pH mediated through kinetic and structural approaches on nuclease P1 is explored. Cosolvents mediated mechanism of stabilization was looked upon using kinetic and thermodynamic approaches. The structural studies were carried out using circular dichroic and fluorescence spectra to estimate the structural alterations upon the interaction of cosolvents with nuclease P1. Thus, the present investigation would provide the information on energetics of the cosolvent-enzyme interactions. This would assist in understanding at a molecular level the forces that are responsible for the stability of the nuclease P1 in presence of cosolvents.

2a. 1. Homogeneity of nuclease P1 enzyme

The homogeneity of the nuclease P1 was evaluated by SDS-PAGE and isoelectric focusing method by determining its pI. Nuclease P1 from *Penicillium citrinum* is a 36 kDa single polypeptide chain with isoelectric point of 4.5 (Fig. 17A & B). The activity was carried out against RNA from yeast as a substrate.

2a. 2. Effect of cosolvents on the activity, stability, structure-function and kinetics of nuclease P1

The effect of selected cosolvents such as glycerol, sucrose, and trehalose on the activity of nuclease P1 was determined using different concentrations (0-30%). Figure 18, shows the relative activity of nuclease P1 in presence of different cosolvents. The native activity of nuclease P1 was taken as 1 fold. The presence of all these cosolvents showed an increment in the activity of nuclease P1. It is clear from the Figure 18 that glycerol was found to be best enhancer of activity of nuclease P1 followed by sucrose and trehalose. Glycerol enhanced the activity of nuclease P1 up to 2.1 fold at 20% concentration. When nuclease P1 was incubated in the presence of different concentration of glycerol, the activity was significantly enhanced than the control at lower concentration, and then shows a gradual decrease over an increase in concentration. Similarly, in the case of sucrose the enzyme was activated up to 20% concentration and above which is not showing any activation and it shows a maximum activation of 2 fold in 5% sucrose concentration (Table 7). In the case of

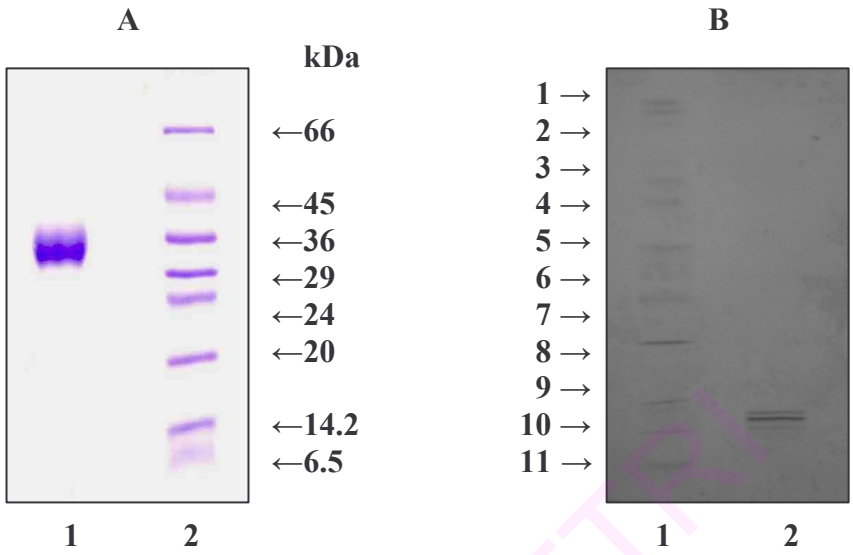

Fig. 17: (A) SDS-PAGE pattern of proteins. (Lane 1) Nuclease P1 from *Penicillium citrinum* (36 kDa). (Lane 2) Standard proteins-BSA (66 kDa), Ovalbumin (45 kDa), Glyceraldyhyde-3-phosphate dehydrogenase (36 kDa), Carbonic anhydrase (29 kDa), Trypsinogen (24 kDa), Trypsin inhibitor (20 kDa), α-Lactalbumin (14.2 kDa) and Aprotinin (6.5 kDa).

(B) Isoelectric focusing pattern of proteins in Ampholine PAG plate pH 3.5-9.5. (Lane 1) Standard pI marker. (1) Trypsinogen (pI 9.30), (2) Lentil lectin-based band (pI 8.65), (3) Lentil lectin-middle band (pI 8.15), (4) Lentil lectin-acidic band (pI 8.15), (5) Myoglobin-basic band (pI 7.35), (6) Myoglobin-acidic band (pI 6.85), (7) Human carbonic anhydrase B (pI 6.55), (8) Bovine carbonic anhydrase B (pI 5.85), (9) β-lactoglobulin A (pI 5.20), (10) Soybean trypsin inhibitor (pI 4.55) and (11) Amyloglucosidase (pI 3.50). (Lane 2) Nuclease P1 from *Penicillium citrinum* (pI 4.5 ± 0.1).

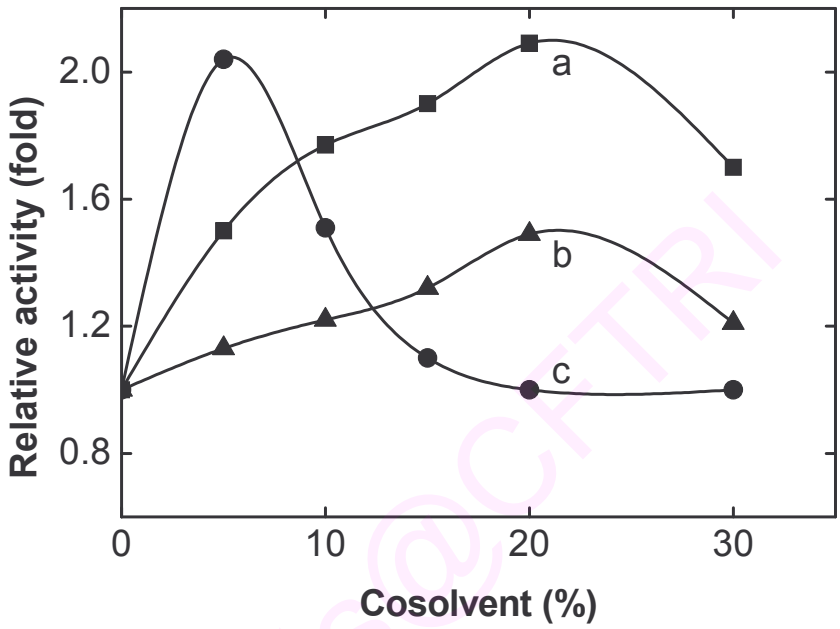

Fig. 18: Relative activity profile of nuclease P1 induced by different concentrations of cosolvents in 0.05 M sodium acetate buffer, pH 5.3. The curve shows the change in enzyme activity in the presence of various concentrations of cosolvents (0-30%). (a) in glycerol, (b) in trehalose and (c) in sucrose.

Table 7: Relative activity of nuclease P1 in absence and presence of various concentrations of cosolvents.

Cosolvent concentration (%) (w/v)		Relative activity (fold)*	
		In presence of cosolvent	After removal of cosolvent
Glycerol	0**	1.0	1.0
	5	1.5	1.0
	10	1.8	1.0
	15	1.9	1.0
	20	2.1	1.0
	30	1.7	1.0
Sucrose	0**	1	1.0
	5	2.0	1.1
	10	1.5	1.0
	15	1.1	1.0
	20	1.0	1.0
	30	1.0	1.0
Trehalose	0**	1.0	1.0
	5	1.1	1.0
	10	1.2	1.0
	15	1.3	1.1
	20	1.5	1.0
	30	1.2	1.0

* Relative activity of nuclease P1 in 0.05 M sodium acetate buffer, pH 5.3 is 1 fold.

** Controls were run parallely in all these experiments.

trehalose the enzyme shows activation up to 30% with a maximum activation of 1.5 fold at 20% concentration of the cosolvent.

The reversibility studies were carried out by using both dialysis and gel filtration method. The enzyme activity was completely regained to its original activity without any fold increase in activity after removal of cosolvents. This result clearly indicates enhancement of nuclease P1 activity by cosolvents is completely reversible. Based on the above results the hierarchy of effectiveness of different cosolvents on the activation of nuclease P1 is in the order (at 20% concentration)

Glycerol > Sucrose > Trehalose

The decrase in the activity at higher concentrations of cosolvents such as, after 5% in sucrose and after 20% both in glycerol and trehalose might be due to steric exclusion of cosolvents. The relatively low enhancement in the activity at higher concentrations of cosolvents could be due to increased viscosity and other biophysical parameters of solution such as surface tension, dielectric constant which could reduce the effective collision between enzyme and substrate molecule (Lee and Lee, 1987).

In order to understand the mechanism of enzyme activation, kinetic experiments were carried out and measured the nuclease P1 activity in presence of above cosolvents as a function of substrate concentration. In presence of all the cosolvents the catalytic constant (k_{cat}) increased from 12 to 27, 19 and 19×10^3 min^{-1} in 20% glycerol, 5% sucrose and 20% trehalose with decreasing K_m values from 1.1 to 0.77, 0.8 and 1 mg resulting in higher catalytic efficiency of cosolvents treated nuclease P1 the k_{cat}/K_m value increased from 11×10^3 to 35×10^3, 24×10^3 and 19×10^3 with above cosolvent concentrations. The activation of nuclease P1 in presence of cosolvents corresponds to the change in the kinetic parameters leading to a conformational change in native state. The conformational flexibility is known to be essential to protein functions such as substrate and ligand binding (Frauenfelder *et al.,* 1991). Presence of osmolytes showed to greatly alter the kinetic properties of enzyme and proteins (Fields *et al.,* 2001). It as also been reported earlier that compatible osmolytes might be expected to affect the association of substrate with enzyme in

several ways, through solvation effects on substrates or enzyme active sites, play a major role in activation and stabilization of enzymes (Cayley *et al.,* 1992).

The effects of cosolvents on the secondary structure of nuclease P1 were examined under optimum conditions, using far UV-CD. Far UV-CD spectra show that the overall molar ellipticity of nuclease P1 was slightly increased in the presence of all cosolvents, without shifts in band positions (Fig. 19). There was no significant change in the structural features of nuclease P1 in presence of cosolvents at optimum condition with slight increase in α-helical content from 32% in control to 39%, 38%, and 39% with slight changes in β-structure from 6% to 5%, 8% and 13% in 20% glycerol, 5% sucrose and 20% trehalose concentration. The aperiodic structure decreased from 62% in control to 56%, 54% and 48% in presence of above cosolvent concentrations. A similar observation has been reported previously for RNase A in the presence of 1 M sucrose (Lee and Timasheff, 1981). Because, the far UV-CD spectrum of a protein arises primarily from the spatial arrangement of amide groups, a small increase in molar ellipticity implies that the native-state ensemble is shifted to favor the protein species with enhanced order of secondary structures (Sreerama and Woody, 1993). Fluorescence emission spectra of nuclease P1 as a function of cosolvents concentrations are studied. At optimum condition the intrinsic fluorescence spectra of nuclease P1 in presence of cosolvents did not shown any effect on both fluorescence emission intensity and position of emission maximum.

The increase in the activity at optimum condition has been related to the hydration of proteins because of the less concentration of nuclease P1 we could not able to study the preferential interaction parameters. Recent studies by Lerbret *et al.,* (2005) suggest that effect of sugars on the hydrogen bonded network of water by Raman scattering and molecular dynamics simulations suggest that they are highly hydrated structures, with trehalose being superior compared with other disaccharides. These cosolvents exhibit greater protective effects in a phenomenon that possibly involves their ability to alter the viscosity and water activity of the medium without bringing any conformational changes in the enzyme. The cosolvents shift the conformational equilibria within the native state ensemble towards more compact, structurally ordered species with lower surface area. Kim *et al.,* (2003) had shown the same effect at optimum condition with sucrose using a model protein.

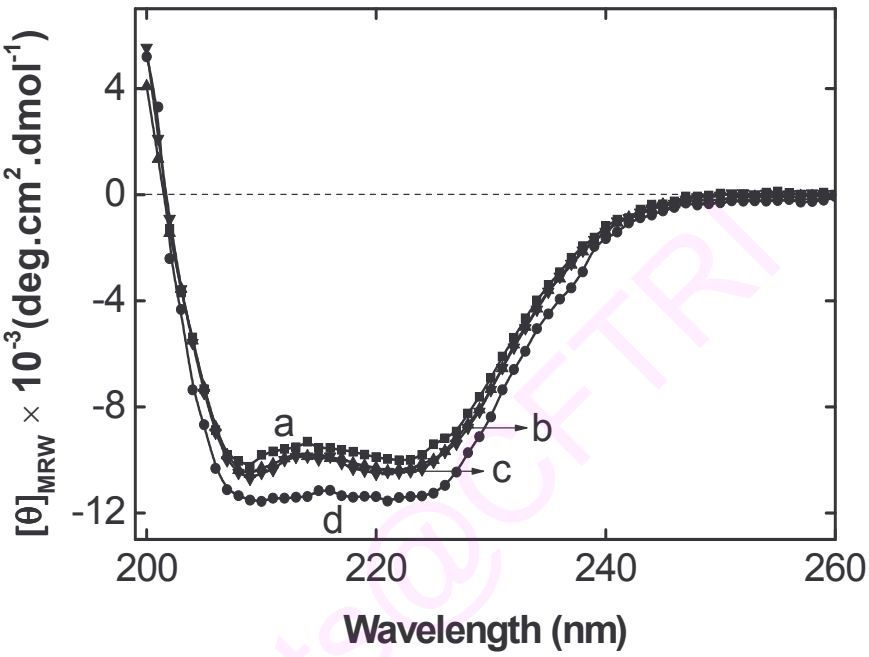

Fig. 19: Far UV-CD spectra of nuclease P1 in the presence of different concentrations of cosolvents in 0.05 M sodium acetate buffer, pH 5.3 at 25°C. (a) control in buffer (0.05 M sodium acetate buffer, pH 5.3), (b) in 5% sucrose, (c) in 20% trehalose and (d) in 20% glycerol.

2a. 3. Effect of cosolvents on the activity, stability, structure-function and kinetics of nuclease P1 at higher temperature

Considerable amount of work has been carried out on the thermal stability of proteins in the presence of a variety of cosolvents. The results of the thermal stability of nuclease P1 in the presence of a series of cosolvents is reported in this chapter using activity, stability, kinetics and structural measurements. Nuclease P1 is ideally suited for understanding the structure-stability relationship in the presence of cosolvents.

The activity profile of nuclease P1 as a function of temperature has shows that sharp decrease in the activity at higher temperature. Nuclease P1 activity measurements were carried out at pH 5.3 and 37°C after incubating the enzyme at 80°C for 20 minutes in the presence of selected cosolvents. Figure 20, shows the residual activity profile of nuclease P1 in presence of cosolvents. Upon incubation at 80°C, activity of about 92% loss was observed in case of native enzyme, but in presence of cosolvents the activity was protected up to 89%. Trehalose was found to be the best stabilizer in protecting the activity of nuclease P1 against thermal inactivation, followed by glycerol and sucrose. About 82 and 89% of the activity was retained in presence of 20 and 30% trehalose respectively. Glycerol found to be less effective when compared to trehalose in protecting the activity of the enzyme and about 79% activity retained at 25% concentration of glycerol. Sucrose is found to be least effective with a maximum 41% retention in the activity at 40% concentration (Table 8).

Enzyme activity was also checked for its reversibility of its original activity after removal of cosolvent by gel filtration on Sephadex G-25 column after exposure to higher temperature. Enzyme fraction eluted in the void volume was used for reversibility studies. From the reversibility studies it is clear that 40% trehalose and 30% glycerol offer maximum protection against thermal inactivation as evidenced by maximum recovery of residual specific activity values of nearly 44 and 37% respectively. Sucrose found to be least effective with a recovery value of 14% at 40% concentration of the cosolvent.

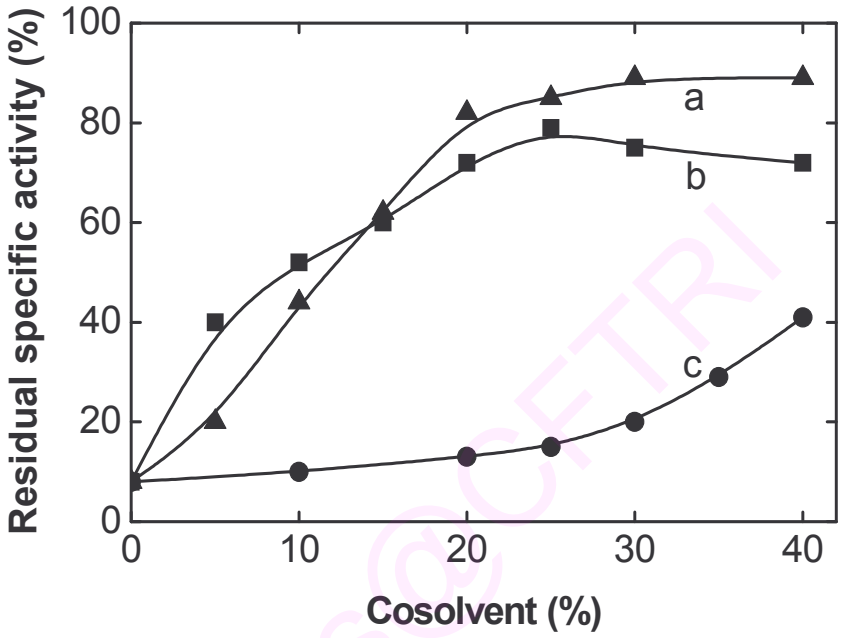

Fig. 20: Thermal inactivation profile of nuclease P1 in the presence of different concentrations of cosolvents in 0.05 M sodium acetate buffer, pH 5.3. The reaction mixture was exposed to 80°C for 20 min with and without cosolvents (0-40%). (a) in trehalose, (b) in glycerol and (c) in sucrose.

Table 8: Residual specific activity of nuclease P1 as a function of cosolvent concentrations after exposure to 80°C for 20 min.

Cosolvent concentration (%) (w/v)		Residual specific activity (%)*	
		In presence of cosolvent	After removal of cosolvent
Glycerol	0**	8 ± 1	0
	5	40 ± 2	16 ± 2
	10	52 ± 2	23 ± 2
	20	72 ± 3	33 ± 2
	25	79 ± 3	37 ± 3
	30	75 ± 3	37 ± 3
Sucrose	0**	8 ± 1	0
	10	10 ± 1	2 ± 0.5
	20	13 ± 1	3 ± 0.8
	25	15 ± 2	4 ± 1
	30	20 ± 2	8 ± 1
	40	41 ± 3	14 ± 2
Trehalose	0**	8 ± 1	0
	5	20 ± 2	8 ± 2
	10	44 ± 2	18 ± 2
	20	82 ± 3	36 ± 3
	30	89 ± 3	45 ± 3
	40	89 ± 3	44 ± 3

* Heat treated nuclease P1 (80°C for 20 min) in 0.05 M sodium acetate buffer, pH 5.3.

** Controls were run parallelly in all these experiments.

To understand the mechanism of stability at higher temperature (80°C for 20 min) the measurements of kinetic parameters (K_m & k_{cat}) of nuclease P1 in the absence and presence of cosolvents were carried out. The data shows a decrease in K_m values from 4 mg in the absence of cosolvents to 1.8, 2.8 and 1.5 mg in presence of 30% glycerol, 40% sucrose and 40% trehalose with increase in k_{cat} values from 0.7 to 10, 5 and 12×10^3 min^{-1}, with increase in k_{cat}/K_m values from 0.18×10^3 to 5.5, 1.8 and 8×10^3 in the presence of 30% glycerol, 40% sucrose and trehalose concentration respectively.

CD spectra of heat denatured nuclease P1 in presence and absence of cosolvents are shown (Fig. 21). At higher temperature there was 10% loss in the secondary structure with shift in band position as compared to that of native condition. In presence of different concentrations of cosolvents both shift in band position and increase in ellipticity values reflects the stabilizing effect of cosolvents with retention of both α-helical and β-sheet content comparable to native enzyme. The α-helical content increases from 22% in control to 33%, 30% and 35% with increase in β-sheet content from 20% in control to 26%, 28% and 43% in 20% glycerol, 30% sucrose and 20% trehalose concentration. The changes observed upon heating are attributable to the loss of secondary structure, leading to temperature-induced unfolding (Arana and Garcia, 1988; Vita *et al.,* 1979).

Figure 22A-C, shows intrinsic emission spectra of nuclease P1 after heat treatment at 80°C for 20 min, in the absence and presence of different concentrations of cosolvents (0-40%). The emission spectra of heat treated nuclease P1 shows emission maximum of 350 nm, compared to native of 340 nm. At higher temperature nearly 50% decreases in the fluorescence intensity and a change in λ_{max} of 10 nm is observed. This shift in the emission maxima of nuclease P1 at higher temperature can be attributed to the conformational changes in the vicinity of solvent exposed tryptophans, presumably due to unfolding of the molecule. In presence of different concentration of cosolvents there was a 100% increase in the fluorescence intensity with no change in emission maximum values.

The stabilization of nuclease P1 as evident by structural measurements has been further confirmed by the measurement of apparent thermal denaturation

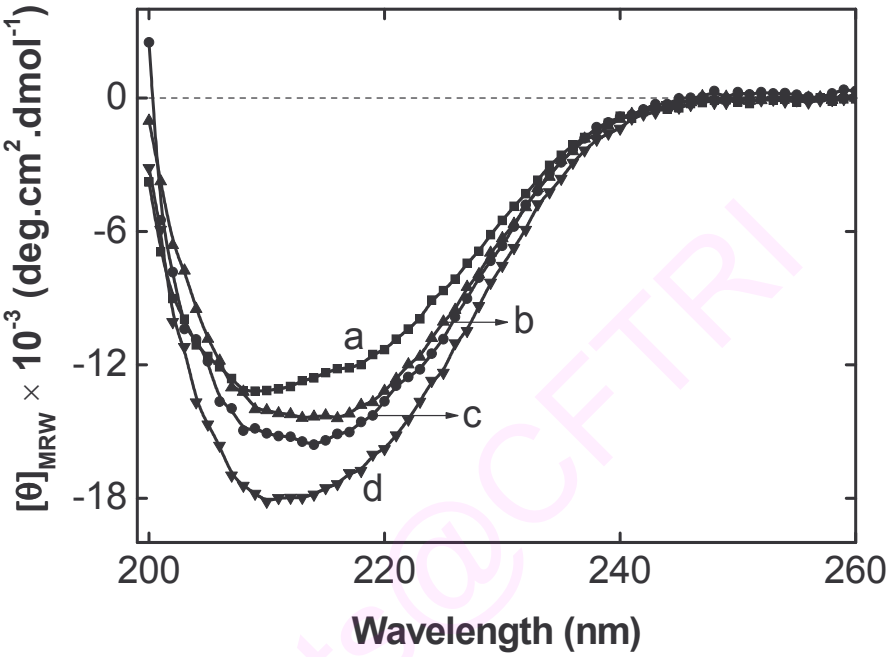

Fig. 21: Far UV-CD spectra of nuclease P1 in the presence of different concentrations of cosolvents in 0.05 M sodium acetate buffer, pH 5.3 after exposure to 80°C for 20 min. (a) control in buffer (0.05 M sodium acetate buffer, pH 5.3), (b) in 30% sucrose, (c) in 20% glycerol and (d) in 20% trehalose.

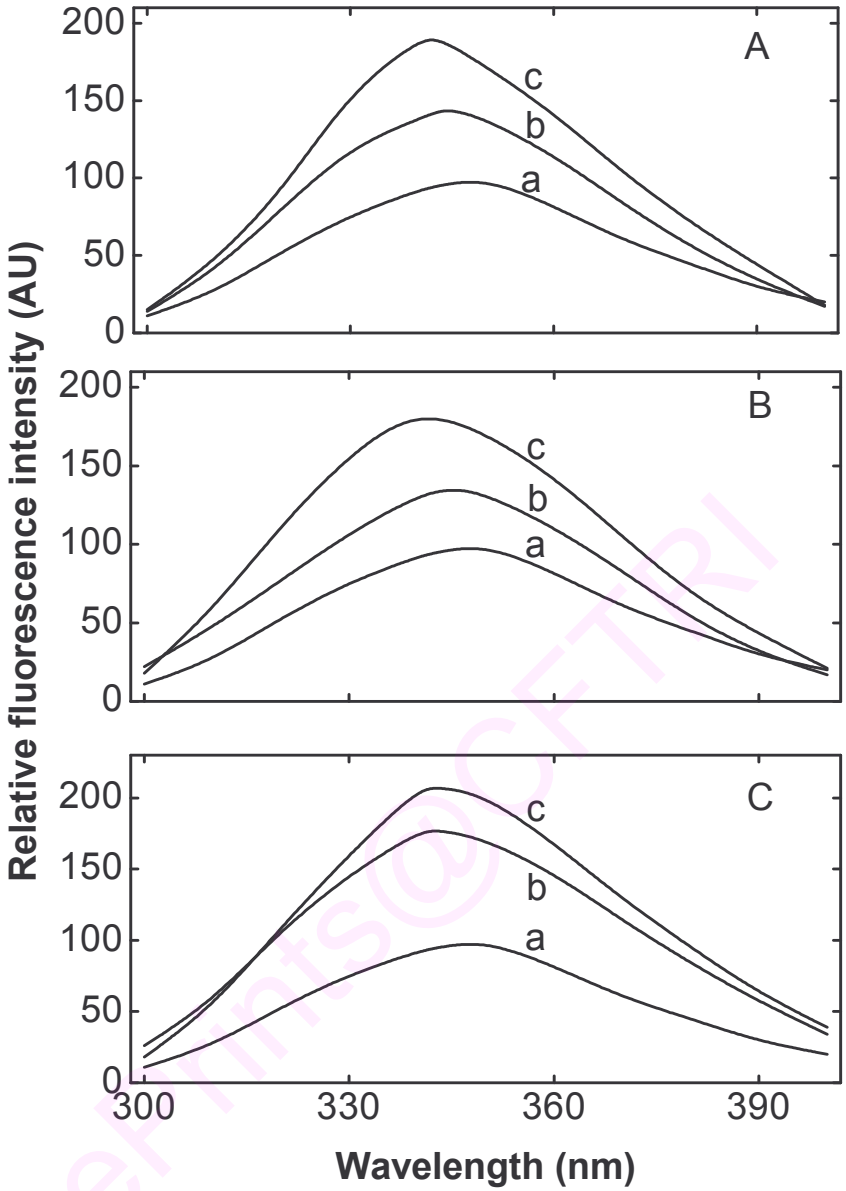

Fig. 22: Intrinsic fluorescence emission spectra of nuclease P1 in the presence of different concentrations of cosolvents in 0.05 M sodium acetate buffer, pH 5.3 after exposure to 80°C for 20 min. Emission spectra recorded over a range of 300-400 nm. (a) control in buffer (0.05 M sodium acetate buffer, pH 5.3), (b) in 20% and (c) in 30% cosolvents. (A) in glycerol, (B) in sucrose and (C) in trehalose.

temperature (T_m) of nuclease P1 both in presence and absence of these cosolvents. Figure 23A-C shows typical thermal transition curves of nuclease P1 in buffer containing various cosolvents. It is evident that the transition curves are shifted to higher temperature with increasing concentration of cosolvents. These cosolvents stabilize the protein structure against thermal denaturation. The apparent T_m shifted from a control value of 75°C to a maximum of 80°C, 84°C and 89°C in presence of 10%, 20% and 30% trehalose concentration respectively. In presence of glycerol apparent T_m rose to 78°C, 81°C, 83°C and 87°C in 10%, 20%, 30% and 40% respectively. Similarly, the increased thermal stability of nuclease P1 was also observed in presence of sucrose and the apparent T_m was found to be 77°C, 79°C, 82°C and 85°C in presence of 10%, 20%, 30% and 40% sucrose respectively.

All these results suggest that the cosolvents exert a stabilizing effect on nuclease P1. Cosolvents were also found to protect the enzyme against thermal inactivation. The protection was a time and concentration dependent phenomenon. The protection of enzymes at higher temperature was due to the ability of the cosolvents to replace water molecules in the medium (Sola-Penna and Meyer-Fernandes, 1998). The mechanism of activity protection was further supported by chaperonins-like action of cosolvents on protein (Kaushik and Bhat, 2003; Martin *et al.,* 1992). In fact, several cosolvents exhibit protective properties, although presenting different degrees of protection (Sola-Penna and Meyer-Fernandes, 1998).

Thermodynamic measurements coupled with biological activity at higher temperature might be a valuable tool for screening additives in enzyme formulations for protection against various degradation mechanisms causing protein conformational destabilization associated with loss of (or decline in) biological activity.

2a. 4. Effect of cosolvents on the activity, stability, structure-function and kinetics of nuclease P1 at alkaline pH

The nuclease P1 activity at pH 9.0 after incubating the enzyme at this pH in the presence of different concentration of cosolvents was determined. Upon incubating, trehalose was found to be the best stabilizer of enzyme against pH induced

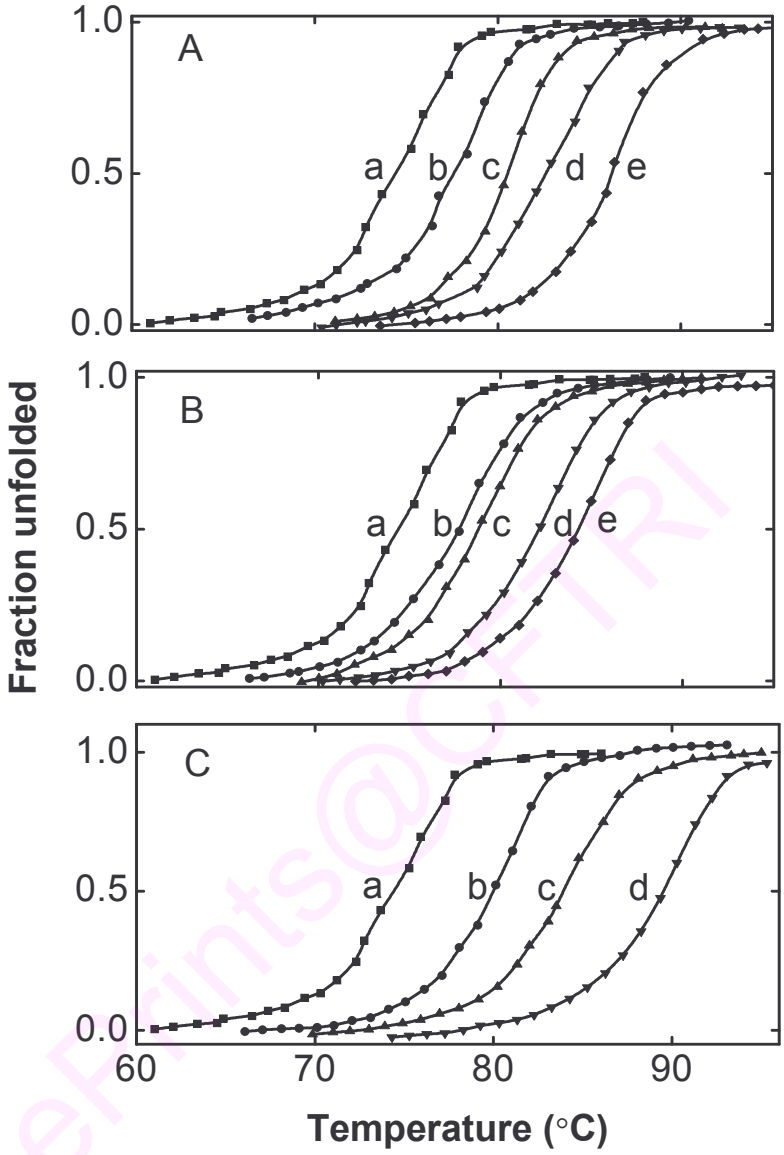

Fig. 23: Apparent thermal denaturation curves of nuclease P1 in the presence of different concentrations of cosolvents in 0.05 M sodium acetate buffer, pH 5.3. The absorption spectra were recorded as a function of temperature at 287 nm. (a) control in buffer (0.05 M sodium acetate buffer, pH 5.3), (b) in 10%, (c) in 20%, (d) in 30% and (e) in 40% cosolvents. (A) in glycerol, (B) in sucrose and (C) in trehalose.

denaturation followed by glycerol and sucrose (Fig. 24). Trehalose retained the activity of nuclease P1 up to 96%, in 30% concentration compared with the retention of less than 5% activity in the absence of cosolvents. Glycerol shows protection of 67% activity at 30% concentration. Compared to trehalose and glycerol, in presence of 10% sucrose the enzyme retained 59% of the activity and found to be less effective (Table 9). From the reversibility studies, it is clear that trehalose and glycerol at 30% concentration offer maximum protection against pH inactivation as evidenced by maximum recovery of residual specific activity values of nearly 22% and 20% respectively. Sucrose found to be least effective with a recovery value of only 14% at 10% concentration.

The pH optimum of nuclease P1 lies between 4.5 and 6.0 and is substrate dependent (Fujimoto *et al.,* 1974c). There are structural reasons which might explain a pH optimum in the acidic region, and it sharply looses its activity once pH increases towards alkaline range. This can be explained in terms of protonated carboxylate pairs of Glu and Asp. They become aprotonated under alkaline conditions; since these amino acid participate in substrate binding, the ionziation affect the kinetics of enzyme catalysis.

The optimum pH of the purified nuclease P1 was found to be 5.3. The enzyme activity decreases in alkaline pH above 7.0. Above pH 7.0, more than 60% of the relative activity was lost. The enzyme lost 95% of the activity at pH 9.0. To understand the mechanism of alkaline stability in presence of cosolvents, kinetic parameter of nuclease P1 activity as a function of substrate concentration at alkaline condition were determined. The control without cosolvents shown the K_m value of 3.3 mg with an apparent k_{cat} value of 0.6×10^3 min^{-1} results in a sharp decrease of the catalytic efficiency of nuclease P1 as compared with the cosolvents treated nuclease P1. By contrast, in the case of cosolvents the K_m value is lower than that of the control with a value of 1.35, 1.57 and 1.61 mg in presence of 30% trehalose, 30% glycerol and 10% sucrose, with an increase in k_{cat} values from 0.6 to 12, 8.3 and 7.5×10^3 min^{-1} in presence of 30% trehalose, 30% glycerol and 10% sucrose. The k_{cat}/K_m value increased from 0.18×10^3 to 4.7, 5.3 and 8.9×10^3 in 10% sucrose, 30% glycerol and 30% trehalose respectively.

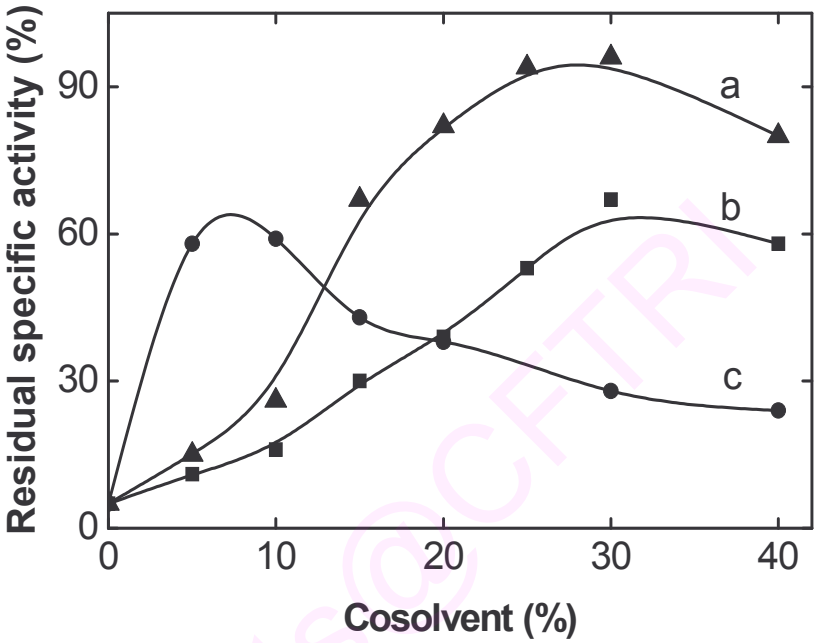

Fig. 24: pH inactivation profile of nuclease P1 in the presence of different concentration of cosolvents in 0.05 M Tris-HCl buffer, pH 9.0. (a) in trehalose, (b) in glycerol and (c) in sucrose.

Table 9: Residual specific activity of nuclease P1 as a function of cosolvent concentrations at pH 9.0.

Cosolvent concentration (%) (w/v)		Residual specific activity (%)*	
		In presence of cosolvent	After removal of cosolvent
Glycerol	0**	5 ± 0.5	0
	5	11 ± 2	3 ± 0.3
	10	16 ± 2	5 ± 0.5
	20	39 ± 3	11 ± 1
	30	67 ± 3	20 ± 2
	40	58 ± 3	18 ± 2
Sucrose	0**	5 ± 0.5	0
	5	58 ± 3	12 ± 2
	10	59 ± 3	14 ± 2
	20	38 ± 2	10 ± 1
	30	28 ± 2	7 ± 1
	40	24 ± 2	7 ± 1
Trehalose	0**	5 ± 0.5	0
	5	15 ± 1	2 ± 0.3
	10	26 ± 2	6 ± 1
	20	82 ± 5	18 ± 2
	30	96 ± 6	22 ± 2
	40	80 ± 4	19 ± 2

* Nuclease P1 in 0.05 M Tris-HCl buffer, pH 9.0.

** Controls were run parallely in all these experiments.

Figure 25 shows the far UV-CD spectra of nuclease P1 in presence of different concentration of cosolvents at pH 9 at 25°C. All the cosolvents were able to protect the secondary structural content of nuclease P1. The α-helical content increases from 28% in control to 36%, 37% and 37% in 20% glycerol, sucrose and 25% trehalose concentration with decrement in β-sheet content from 14% to 8%, 5% and 4% with above cosolvent concentrations respectively with minor changes in aperiodic structure.

Figure 26A-C shows intrinsic fluorescence spectra of nuclease P1 in presence and absence of cosolvents at pH 9.0. At alkaline pH shift was observed in the emission maximum with decrease in emission intensity. As shown in Figure 26 the emission maximum of cosolvents treated enzyme shows blue shift with increase in fluorescence intensity close to native enzyme. This suggests that cosolvents protected the conformation of enzyme into a more compact state.

The above results in different conditions clearly suggest that all cosolvents used were stabilizing the nuclease P1 activity at different conditions. Since the stability of proteins results from a large number of counteracting enthalpic and entropic contributions which favor the folded state as compared to the unfolded state. There are several reports indicating that these cosolvents exert their effects on protein stability and this has given rise to a number of concepts important in discussing and investigating cosolvent-protein interaction (Bolen and Baskakov, 2001; Street *et al.,* 2006; Schellman, 2003; Timasheff, 2002b; Tiwari and Bhat, 2006).

It is well documented by hydrogen exchange studies that, the native conformation of protein is flexible and does not exist as discrete single structure (Englander *et al.,* 1980; Wang *et al.,* 1995). Rather, fluctuations from the most compact form of the protein will transiently exposed the protein core to solvent. According to Lee and Timasheff, (1981) sucrose should favor the most compact protein conformation, even under nondenaturing conditions. In case of recombinant human interferon with sucrose (Kendrick *et al.,* 1997) it has been shown that preferential exclusion of cosolvents is the main factor which increases the protein chemical potential within the native state, favoring more compact conformations. This

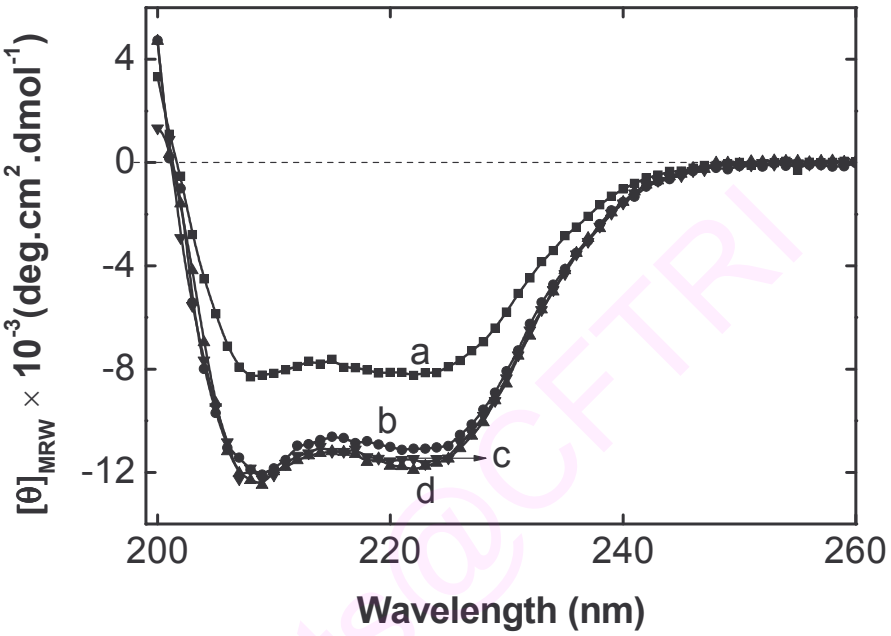

Fig. 25: Far UV-CD spectra of nuclease P1 in the presence of different concentrations of cosolvents in 0.05 M Tris-HCl buffer, pH 9.0 at 25°C. (a) control in buffer (0.05 M Tris-HCl buffer, pH 9.0), (b) in 20% glycerol, (c) in 20% sucrose and (d) in 25% trehalose.

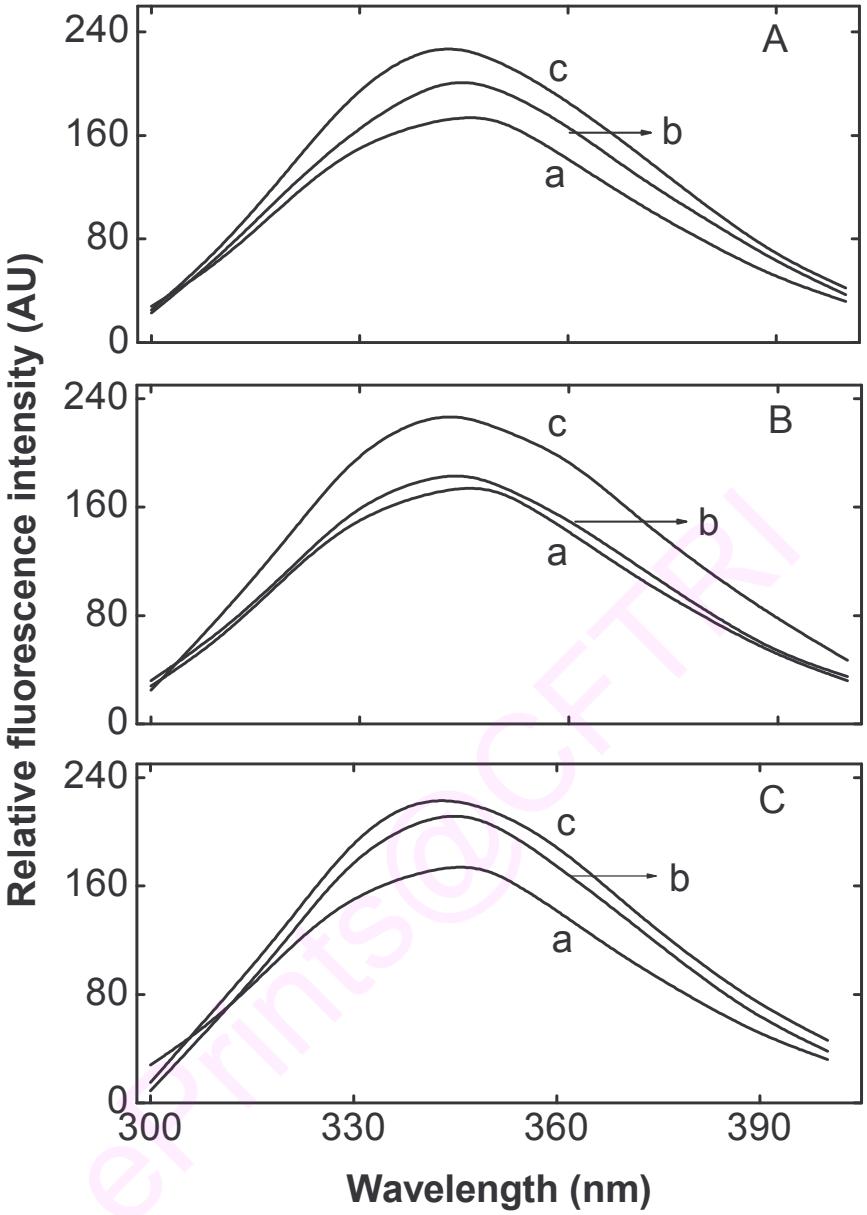

Fig. 26: Intrinsic fluorescence emission spectra of nuclease P1 in the presence of different concentrations of cosolvents in 0.05 M Tris-HCl buffer, pH 9.0. Emission spectra recorded over a range of 300-400 nm. (a) control in buffer (0.05 M Tris-HCl buffer, pH 9.0), (b) in 20% and (c) in 30% cosolvents. (A) in glycerol, (B) in sucrose and (C) in trehalose.

provides a clear discussion of this phenomenon based upon the Gibbs adsorption isotherm and the Wyman linkage relation.

In case of ribonuclease A (Wang *et al.,* 1995), it is documented that dynamic structural fluctuations which lead to transient increase in protein surface area and allow hydrogen-deuterium exchange, are attenuated in the presence of sucrose. Here we apply this concept on the effects of cosolvents on the conformational changes that must occur during enzyme function and stabilization. This can be explained from the kinetic approaches which have indicated lower K_m and higher k_{cat} values in the presence of cosolvents.

Activity retention at higher temperature and alkaline pH is seen to depend upon the cosolvents used. The mechanism of their action is not completely understood yet, but there are several hypotheses leading to thermodynamic stabilities. The protein-stabilizing action of cosolvents has been explained in terms of free energy changes (Arakawa and Timasheff, 1982a; Bolen and Baskakov, 2001; Kaushik and Bhat, 2003; Timasheff, 2002a; Xie and Timasheff, 1997c). Most of the hypotheses are based on the preferential hydration of proteins, which means that in a protein-water-cosolvent ternary system, the cosolvent is preferentially excluded from the immediate proximity of the protein (Arakawa and Timasheff, 1982a; Timasheff, 2002a).

Compared to sucrose both trehalose and glycerol offered maximum protection of enzyme activity, both at higher temperature and alkaline pH. This can be explained based on their mechanism. Thermodynamically unfavorable interaction tends to minimize the surface of contact between proteins and glycerol and in this way stabilizes the native structure of globular proteins in general (Gekko and Timasheff, 1981b). Trehalose is known to stabilize the enzyme against thermal and alkaline denaturation (Kaushik and Bhat, 2003). Because, it fits into the network of the respective hydrogen bonds while they avoid interaction with hydrophobic side chains and peptide groups by exclusion (Liu and Bolen, 1995).

The studies presented in this chapter clearly demonstrate that cosolvents could be used to increase the stability and activity of nuclease P1 and also to prevent the permanent inactivation of enzyme subjected to extreme conditions. One can take

advantage of enzyme specificities under different conditions in presence of cosolvents to catalyze reactions that could be limitation otherwise. Therefore, the present study from the activity, stability, kinetic measurements of the nuclease P1 at optimum, high temperature and alkaline pH conditions and structural determination in presence of cosolvents using thermal denaturation, fluorescence and secondary structural studies clearly show that cosolvents can induce the formation of intermediate state which is expected to have characteristics of molten globule state, the effect being stronger with increasing the concentration of cosolvents. This molten globule state is useful for converting a wide variety of enzymes to thermo, pH stable and thermoactive ones for a variety of applications in biological, medical and biotechnological fields.

2b. Effect of denaturants on the activity, stability, structure-function and kinetics of nuclease P1

2b. Effect of denaturants (Urea and/or GuHCl) on the structure, function, kinetics and activity of nuclease P1

Several factors contribute to the low activity of enzymes, which includes solvent-induced structural changes that affect the enzymes active site, lyophilization-induced dehydration of critical regions of the enzyme, ground-state stabilization of hydrophobic substrates and poor compatibility between the solvent and transition state (Desai and Klibanov, 1995; Ryu and Dordick, 1992). Many strategies have been used to increase enzyme activity, such as protein engineering, molecular imprinting and addition of cosolvents, organic solvent to increase the activity (Almarsson and Klibanov, 1996; Kuchner and Arnold, 1997; Russell and Klibanov, 1988; Sears *et al.,* 1994). The activity of enzymes is strongly dependent on their conformational integrity (Tsou, 1993), and some of the enzymes exhibit, remarkable and unique properties such as enhanced thermal stability, altered substrate specificity, enantiomeric specificities and molecular memory (Klibanov, 1995).

Urea and/or GuHCl are most commonly used chemical denaturants of proteins, and numerous conformational/unfolding studies of proteins have been carried out using these denaturants (Santoro and Bolen, 1988). In most of the cases inactivation of the enzymes occurs at lower denaturant concentrations than required to bring about unfolding of the protein (Tsou, 1986). However, low concentration of urea and/or GuHCl treated enzyme shows marked increase in activity and is reported in this chapter. The present study tries to analyze the mechanism of higher activity of nuclease P1 in presence of urea and/or GuHCl at low concentration.

In the present study, an attempt has made to study the effect of urea and/or GuHCl on the activity of nuclease P1 and found that the activity was enhanced at lower concentrations. It is interesting to investigate the activity at low concentration of denaturants. The availability of the data has been far less and few references have shown that such enhancement of activity at low concentration of denaturant do occur (Fan *et al.,* 1995; 1996). The influence of urea and/or GuHCl on nuclease P1 was studied in detail using enzyme kinetic, structure and stability studies to gain insight

into the mechanism of stability of the enzyme in low concentrations of urea and/or GuHCl and its mechanism of activation.

The activity profile of nuclease P1 as a function of urea and/or GuHCl concentration is shown in the Fig. 27A & B. The results show the activity enhancement of nuclease P1 in presence of these denaturants at lower concentrations. The fold of enhancement in the activity is dependent on the concentration of urea and/or GuHCl used. The concentration between 0.02-1 M of GuHCl and 0.25-4 M of urea the nuclease P1 activity was significantly enhanced. At 1 M urea and 0.05 M GuHCl, the nuclease P1 activity reached its maximum of nearly 3.9 fold and 3 fold respectively (Table 10). The enzyme activity was decreased above 1 M GuHCl and 4 M urea in a concentration dependent manner. The enzyme activity was completely lost as the concentration of GuHCl reached greater than 2 M. But the enzyme still maintains 50% of the activity at 5 M urea but the activity becomes zero when the concentration of urea reaches above 8 M. These result clearly evident that nuclease P1 activity was enhanced at low concentration of urea and/or GuHCl. In presence of DNA as substrate, the activity of nuclease P1 (control) was less as compared to RNA as a substrate. The control show the activity of 67% (0.67 fold) in presence of DNA where RNA was taken as 100% (1 fold). In presence of different concentrations of urea or GuHCl the nuclease P1 activity with DNA as a substrate show the same effect as evidenced from RNA as a substrate and this clearly indicates that urea and/or GuHCl enhances the activity of Nuclease P1.

To follow up the activity enhancement at low concentration of denaturants, the kinetic properties of the nuclease P1 in the absence or presence of denaturants were determined using Lineweaver-Burk (or) double-reciprocal plot. In the absence of denaturants the K_m was 1.1 mg with catalytic constant (k_{cat}) of 12×10^3 min^{-1}. The K_m decreased to 0.86 mg and 0.60 mg with increment in k_{cat} values to 39×10^3 min^{-1} and 55×10^3 min^{-1} in presence of 0.05 M GuHCl and 1 M urea and k_{cat}/K_m values also increased from 11×10^3 to 45×10^3 and 91×10^3 with above concentration respectively. These results such as decrease in K_m and increase in the catalytic constant suggest that nuclease P1 is achieving a maximal catalytic efficiency in presence of low concentration of urea and/or GuHCl.

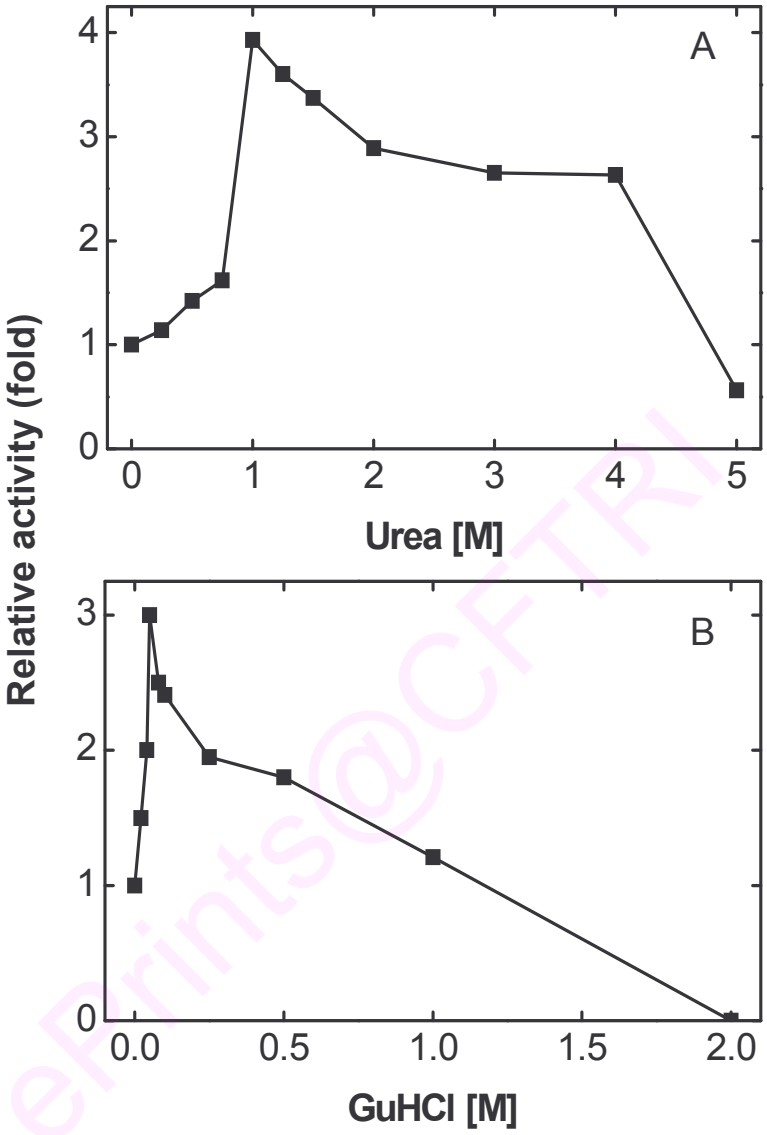

Fig. 27: Relative activity profile of nuclease P1 induced by different concentrations of denaturants in 0.05 M sodium acetate buffer, pH 5.3. The curve shows the change in enzyme activity in the presence of various concentrations of denaturants. (A) in urea and (B) in GuHCl.

Table 10: Relative activity of nuclease P1 in absence and presence of various concentrations of denaturants.

Denaturant concentration (M)		Relative activity (fold)*	
		In presence of denaturant	After removal of denaturant
Urea	0**	1.0	1.0
	0.25	1.1	1.0
	0.50	1.4	1.0
	0.75	1.6	1.0
	1.00	3.9	1.1
	1.25	3.6	1.0
	1.50	3.4	1.0
	2.00	2.9	1.0
GuHCl	0**	1.0	1.0
	0.02	1.5	1.0
	0.04	2.0	1.1
	0.05	3.0	1.0
	0.08	2.5	1.0
	0.10	2.4	1.0
	0.25	2.0	1.0
	0.50	1.8	1.0
	1.00	1.2	1.0

* Relative activity of nuclease P1 in 0.05 M sodium acetate buffer, pH 5.3 is 1 fold.

** Controls were run parallely in all these experiments.

To follow the activity enhancement at low concentration, activity of nuclease P1 was determined in absence and presence of detergent and metal ions. The detergent, Triton X-100 had a negative effect on enzyme activity with about 28% and 35% reduction in 1% and 2% Triton X-100 respectively. Both copper and cobalt decreases the nuclease P1 activity. In case of copper ($CuCl_2$) the enzyme loses nearly 65% of activity at 1×10^{-3} M concentration. In presence of 1×10^{-3} M cobalt ($CoCl_2$) concentration nearly 29% of the enzyme activity is lost. Control activity is assigned one hundred percent in the absence of metal ions. From the results it is clear that urea and/or GuHCl treated enzyme in presence of metal ions still maintains the activity enhancement. In presence of cobalt at 1 mM concentration the enzyme loses only 29% of its residual activity, with urea and/or GuHCl the enzyme is able to maintain its activity enhancement. Compared to cobalt in presence of copper the enzyme loses 65% of its residual activity and the activity enhancement with urea and/or GuHCl is retained.

From the reversibility studies both by dialysis and Hummel-Dreyer gel filtration method the enzyme activity is returned to normal state without any fold increase in the activity, once the urea and/or GuHCl is removed from the system clearly indicating the complete reversibility of activity. To see long term stability of the enzyme in presence of denaturants, enzyme activity measurements are performed in presence of denaturants as a function of time and checked relative activity of enzyme for control as well as in presence of urea and/or GuHCl for 30 min, 24 h and 48 h. The results of stability study clearly indicate that the activated enzyme is stable up to 48 hours. In lower concentrations of these two denaturants the relative activity does not change even at 48 h.

A far UV-CD spectrum was used to monitor conformational changes of nuclease P1 as a result of addition of urea and/or GuHCl addition. Figure 28 shows the far UV-CD spectra of nuclease P1 in presence of urea and/or GuHCl, and the enzyme exhibited two minima at 222 and 208 nm. In the presence of denaturants shows a small increment in the ellipticity values in the region of 208-223 nm. There was no significant change in the structural features of nuclease P1 in presence of denaturants. In case of 1 M urea the enzyme shows no major conformational changes.

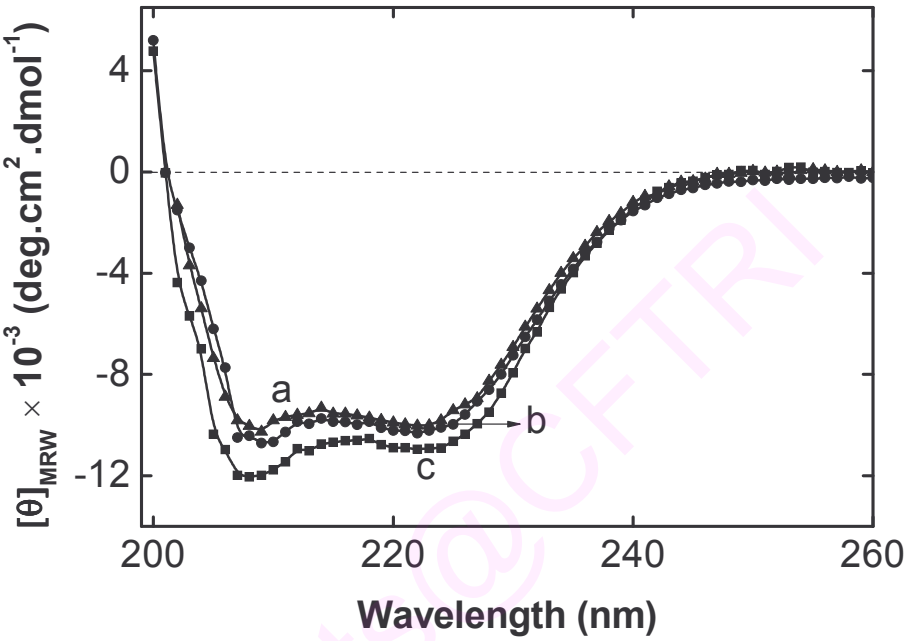

Fig. 28: Far UV-CD spectra of nuclease P1 in the presence of different concentrations of denaturants in 0.05 M sodium acetate buffer, pH 5.3 at 25°C. (a) control in buffer (0.05 M sodium acetate buffer, pH 5.3), (b) in 0.05 M GuHCl and (c) in 1 M urea.

Similarly in 0.05 M GuHCl the enzyme shows 34% α-helical content and slight decrease in β-structures from the control value 6% to 5% in 0.05 M GuHCl and 3% in 1 M urea with no change in aperiodic structure from 62% to 61% and 63% with above concentration. Micro environmental changes near the active site of enzyme or in its neighborhood would generally bring about such alterations. Such changes which are rather unusual for nuclease P1 in presence of low concentration of denaturants gave the importance of this observation.

The effect of urea and/or GuHCl on the fluorescence emission spectra of nuclease P1 is shown in Fig. 29A & B. The spectra clearly indicate the presence of two transitions, one at lower concentration and the other one at higher concentration. In case of GuHCl the transition point was sharp there was a 100% increase in fluorescence intensity at lower concentration, indicating that surface tryptophans are in a hydrophobic environment. In case of urea only 40% increase in intensity was observed without any change in the emission maximum at lower concentration. But at higher concentrations (Urea>1.5 M and GuHCl>0.1 M) there was decrease in intensity without any change in the emission maxima. These data suggest the possibility of more microenvironment changes and the tryptophanyl molecule at these low concentrations of denaturants making restricted exposure of aromatic amino acid to the bulk solvent.

The extent of change in the conformation of nuclease P1 resulting from the additives action was evaluated further through measurement of the apparent thermal denaturation temperature of enzymes in presence and absence of different concentration of urea and/or GuHCl. Figure 30A & B show the plot of fraction unfolded verses temperature and the apparent T_m were calculated. The Figure 30A & B indicates a progressive increase in the apparent T_m values of nuclease P1 at lower concentration of urea and/or GuHCl. The apparent denaturation temperature shifted from 75°C to 77°C, 74°C and 73°C in 0.5 M, 1 M and 2 M urea respectively. In presence of GuHCl the apparent T_m value increased to 79°C and 80°C at 0.05 M and 0.1 M concentration respectively from the control value of 75°C. These results indicate that the transition is higher for nuclease P1 in the presence of lower

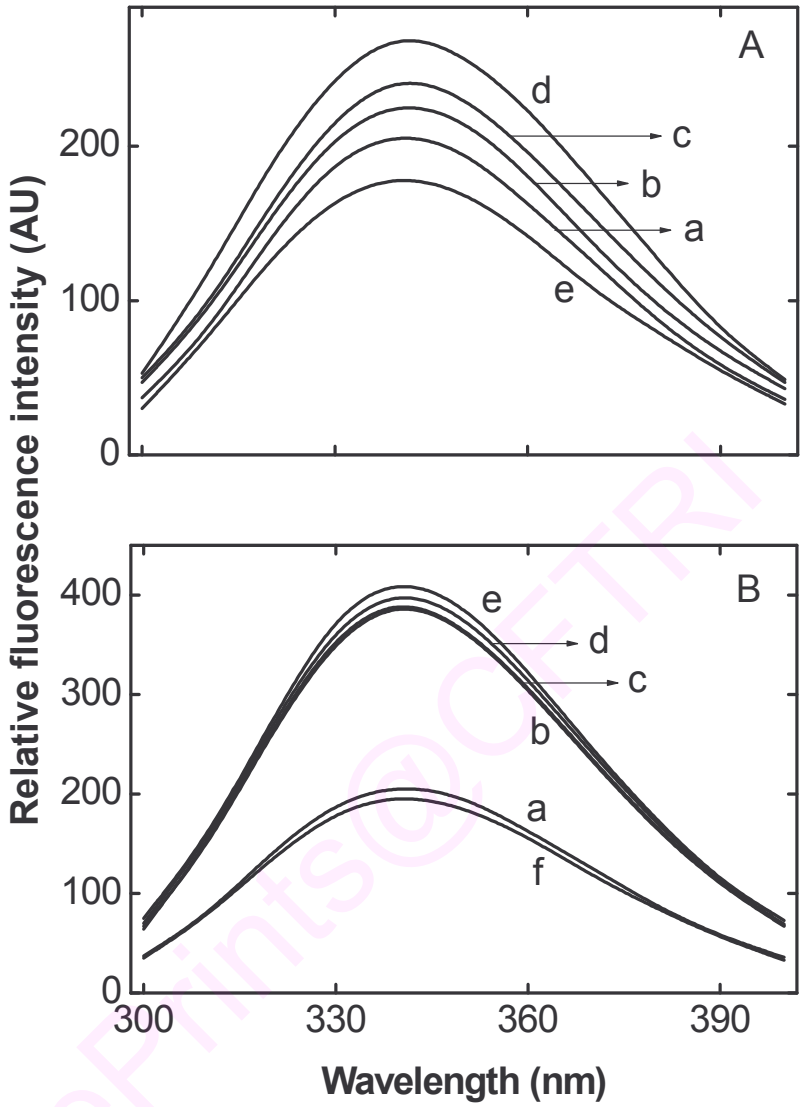

Fig. 29: (A) Intrinsic fluorescence emission spectra of nuclease P1 in the range of 300-400 nm in the (a) absence and presence of (b) 0.5 M, (c) 0.75 M, (d) 1 M and (e) 2 M urea. (B) In the (a) absence and presence of (b) 0.02 M, (c) 0.04 M, (d) 0.05 M, (e) 0.08 M and (e) 0.1 M GuHCl in 0.05 M sodium acetate buffer, pH 5.3.

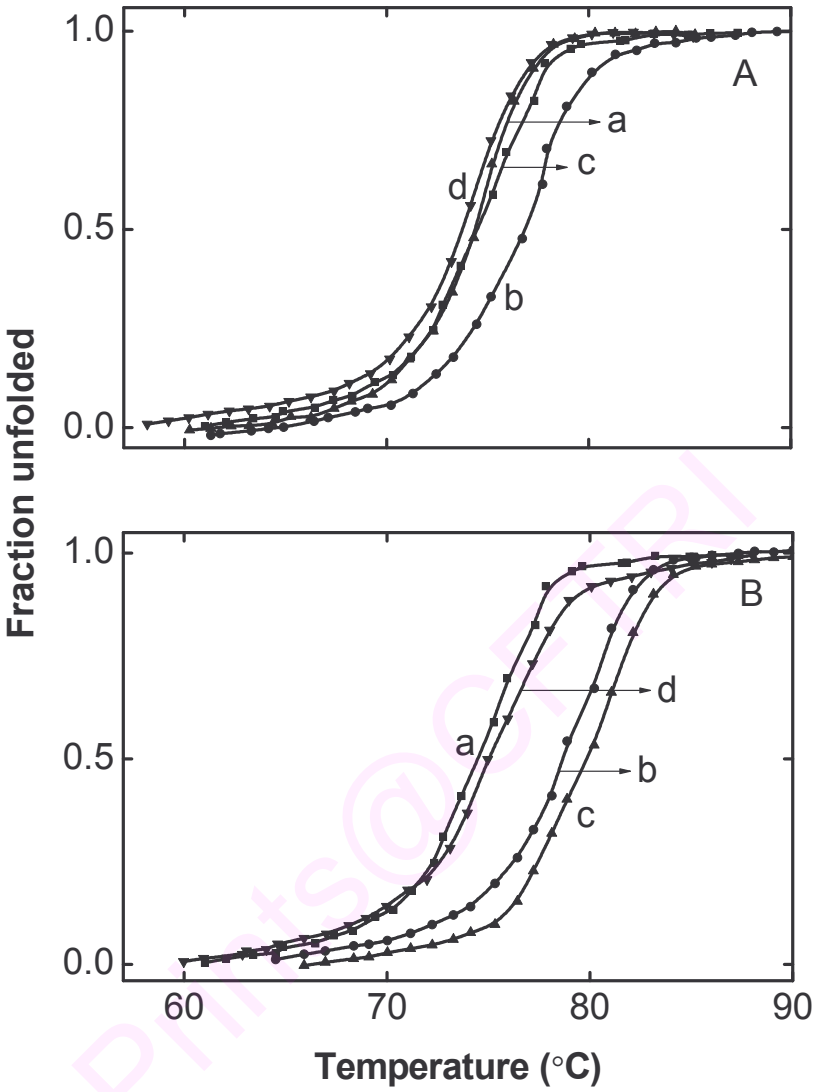

Fig. 30: (A) Apparent thermal denaturation curves of nuclease P1 in the (a) absence and presence of (b) 0.5 M, (c) 1 M and (d) 2 M urea. (B) In the (a) absence and presence of (b) 0.05 M, (c) 0.1 M and (d) 0.5 M GuHCl in 0.05 M sodium acetate buffer, pH 5.3. The absorption spectra were recorded as a function of temperature at 287 nm.

concentration of urea and/or GuHCl. At the concentration above 1 M in case of urea and 0.5 M GuHCl the T_m of the enzyme decreases.

The intermediate state may help in activation process and also stabilization of microenvironment of the energetically favorable state. In many enzymes and proteins the molten globule state has been proposed to be involved in a number of such physiological processes, such as release of protein ligands, protein recognition by chaperones, and protein translocation across membranes (Takashi *et al.,* 2003). Human acidic fibroblast growth factor found to form a stable equilibrium intermediate accumulated maximally at 0.96 M GuHCl resembling a molten globule state as examined by CD, fluorescence, and NMR (Samuel *et al.,* 2000). These are characterized by the existence of expandable intermediate as studied in case of heat shock protein (Palleros *et al.,* 1993), increased thermal stability in case of Rnase T1 (Mayr and Schmid, 1993), and possibility of hydration of exposed hydrophobic group in case of tubulin (Guha and Bhattacharyya, 1995).

The present results have proved that nuclease P1 activity was increased nearly 3.9 fold and 3 fold in presence of 1 M urea and 0.05 M GuHCl, mainly because of an increase in catalytic constant (k_{cat}) of the activated enzyme. Moreover, an increase in binding affinity of the activated state for the substrate, as shown by the decreased K_m value also contribute to the enhancement of nuclease P1 activity by urea or GuHCl at lower concentrations. In case of higher concentrations of GuHCl (>2 M) the enzyme is completely inactivated but the enzyme still maintains 50% of the activity at 5 M urea but the activity becomes zero when the concentration of urea reaches above 8 M. However many enzymes are completely denatured and devoid of activity in 8 M urea and GuHCl (Santoro and Bolen, 1988; Sears *et al.,* 1994).

At higher concentrations, denaturants are known to disrupt protein/enzyme secondary and tertiary structures, but in our study we are using the denaturants at low concentration. For activity enhancement studies only low concentration of denaturants employed and at this concentration, denaturants will not induce any structural changes in the RNA. In the case of DNA also the enhancement of activity was similar to RNA and it clearly indicates that these denaturants at low concentration does not have any effect on the structural changes in RNA.

There are various reports existing in the literature pertaining to metal ions inhibition on nucleases from potato tubers, *B. subtilis* and pea seeds (Nomura *et al.,* 1971; Kanamori *et al.,* 1973; Naseem and Haidi, 1987). Recently, Qing *et al.*, 2006, also studied the effect of various detergents and metal ions on the activity of nuclease P1 which was isolated and purified from *Penicillium citrinum*. Their results indicated that Triton X-100 had a negative effect on enzyme activity and both copper and cobalt inhibits enzyme activity which was similar to our results and also our studies shows urea and/or GuHCl treated enzyme in presence of these two metal ions still maintains the activity enhancement up to different extents.

If there is an irreversible conformational change as a result of molten globule associated stable state as a result of exposure in GuHCl and/or urea separately, then upon removal of urea and/or GuHCl the original activity of the enzyme must come back to its original. This is exactly the result that has been obtained after reversibility experiments which we have done. The activities of control nuclease P1 before exposure to denaturants and upon exposure to 0.05 M GuHCl and 1 M urea and subsequent removal of urea and/or GuHCl (both by dialysis and Hummel-Dreyer column) were identical, within the experimental errors. The k_{cat}/K_m ratio was in the range of $11\text{-}12\times10^3$ clearly indicates the reversibility of the process of activation of nuclease by urea and/or GuHCl.

There are many reports on activation of other enzymes by denaturants, associated with an increment in the catalytic constant (Fan *et al.,* 1995; 1996; Tsou, 1993). The present study shows that enzyme is stable in its activated form as evidenced by circular dichroism and fluorescence experiments suggesting further internalization of surface tryptophans in the hydrophobic environment at low concentration of these denaturants and formation of a more energetically favorable structure. In the case of adenylate kinase and dihydrofolate reductase (Fan *et al.,* 1995; 1996), no significant change of the overall structure of the molecule is observed in presence of denaturants as monitored by CD and fluorescence spectra. Therefore, the activation of these enzymes is supposed to be caused by an increase in the conformational flexibility at the active site. The activated enzyme loses its activity once the concentration of urea and/or GuHCl reaches 5 and 2 M respectively. X-ray

crystal structure of Nuclease P1 shows that flexibility in the substrate binding and active site is very important for activity. Nuclease P1 binding site remote from the active site, which is composed of Tyr 144, Tyr 155 and Asp 146 and it display low flexibility in terms of adaptation to different substrates (Volbeda *et al.,* 2004). It would seem likely, therefore, that the active site of nuclease P1 is situated in a region having higher flexibility for change in the microstructure.

From the secondary structural analysis, minor changes are observed in the secondary structural content, except with slight increase in α-helix from 32% in absence of denaturants to 34% in presence of urea and GuHCl and a decrease in β sheet content from 6% to 3% and 5% in presence of urea and/or GuHCl respectively. The fluorescence studies show that at lower concentration relative fluorescence intensity is increasing, which indicates that hydrophobic amino acids are not exposing to the bulk solvent. In case of ATPase activity and structure of soluble mitochondrial F1 it is shown that GuHCl induces a sequence of events that initially comprise subtle structural perturbation at low concentration of denaturants (Puyou *et al.,* 2001).

Interaction of urea and/or GuHCl with several proteins has been monitored by partial specific volume measurement and calculations of various preferential interaction parameters based on firm principles of thermodynamics (Prakash *et al.,* 1981; Prakash, 1982) and the detailed studies with more emphasis on the intermediates at lower concentrations of urea are earlier reported in case of human serum albumin and papain (Muralidhara and Prakash, 1997; Sathish *et al.,* 2002). These results suggest that there are two major structural features in presence of denaturants as observed in case of nuclease P1 also. One molten globule state of the enzyme at lower concentration of denaturants, and second the unfolding process of the enzyme leading to the denatured state at higher concentration of denaturants. Many of the well-established characteristics of the molten globule state of the protein are observed at lower concentration of denaturants (Kuwajima, 1989; Uversky *et al.,* 1992). The results of far UV-CD studies of Nuclease P1 in presence of low concentrations of denaturants show increase in rotations at 222 nm suggesting increment of α-helical structure. Further increase in apparent T_m is also observed in thermal denaturation measurements. This molten globule state has been captured in some proteins at low concentration of denaturants (Rose *et al.,* 2006). In this case also

the increase in α-helical content and apparent T_m suggests such a state of the molecule.

Hence the several folds increase in the activity of the enzymes in the presence of urea and/or GuHCl at low concentrations is proposed to be caused by conformational flexibility probably at the active site and the formation of molten globule like structure of enzyme. The results observed with nuclease P1 in terms of activity measurement, kinetic, CD, fluorescence and thermal denaturation studies in the present investigations which substantiate the formation of an intermediate molten globule like state of nuclease P1 in these denaturants. Since nuclease P1 and related enzymes are widely used by molecular biologists as an analytical tool, addition of low concentration of urea and/or GuHCl can enhance its activity in many of the reactions at these low concentrations.

CHAPTER – 3

STUDIES ON LIPASES

3a. Effect of selected ligands such as metal ions and azadirachtin on the inhibitory effect on rice bran lipase

3a. Effect of selected ligands such as metal ions and azadirachtin on the inhibitory effect on rice bran lipase

Lipases are versatile enzymes that catalyze the hydrolysis of ester linkages, primarily in neutral lipids such as triglycerides. They hydrolyze the acyl chains either at primary or secondary positions (Ransac *et al.,* 1990). In plants, the regulation, in some cases the location and the exact physiological roles of lipases are not very clear. They are known to play an essential role in the mobilization of seed-storage lipids to support germination and post-germinative embryonal growth.

Rice bran is a by product of the milling process. It constitutes nearly 7-8% of the total rice grain. When bran layers are removed from the endosperm during the milling process, the individual cells are disrupted and the rice bran lipids come into contact with highly reactive lipases. These enzymes are both endogenous to the bran and of microbial origin and initiate hydrolytic deterioration of kernel oil (Champagne *et al.,* 1992). Rice (*Oryza sativa*) bran oil is typically an oleic-linoleic-type fatty acid, and its physical-chemical properties qualify it for good quality edible oil. However, complete utilization of bran oil suffers from the fact that there is a large accumulation of free fatty acids (FFA), which has been attributed to the presence of lipase activity (Funatsu *et al.,* 1971). The lipase is present in the bran region of the grain. Most of the lipids in rice grains are concentrated in outer layers viz. aleurone layer and bran. Rice bran is prone to lipase action which increases free fatty acid content in the oil, which becomes unsuitable for processing to edible oil (Aizono *et al.,* 1976; Shastry and Raghavendra Rao, 1976).

Several methods have been applied to arrest the lipolysis in the bran including; heating, treatment with hydrochloric acid, exposure to microwave irradiation, effect of chemical inhibitors and ohmic heating, only bring about partial inactivation (Nasirullah *et al.,* 1989; Rao *et al.,* 2004). In order to optimize the suitable method for lipase inactivation there is a need to understand the mechanism of inactivation of lipase using different type of inhibitors. In the present study, interaction of rice bran lipase with selected ligands such as metal ions and azadirachtin has been carried out with a view to elucidate the mechanism of inactivation.

3a. 1. Effect of metal ions on rice bran lipase activity

3a. 1. Effect of metal ions on rice bran lipase inhibitory activity

Metal ions have been referred to as super acids and the majority of these functions as key components of essential enzymes which perform vital roles in biological systems. It is well established that metal ions are better catalysts than mineral acids under biological conditions and are normally effective in trace amounts. Therefore, it has been considered worthwhile to use the metal ions for the inactivation of rice bran lipase as no such work has been reported on this aspect, with an ultimate goal of exploiting the potential of metal ions for producing edible grade oil from rice bran. Keeping above ideas we have screened several metal ions like zinc, copper, ferrous chloride, lithium and non metal such as selenium on the activity of RBL.

Among all the metal ions only lithium and selenium shows inhibitory effect at lower concentration. Selenium is an essential trace element for human health, and has received considerable attention for its possible role as an effective naturally occurring, anticarcinogenic agent and exerts its biological effects either directly or by being incorporated into enzymes and other biological proteins (Ip, 1998). Selenium appears to modulate cellular activities presumably by acting on proteins important for signal transduction by suppressing the C-Jun N-terminal kinase/stress-activated protein kinase (Park *et al.,* 2000). Selenium dioxide is considered as acidic oxide, it dissolve in water to form selenous acid. Although selenium it is toxic in large doses, it is essential micronutrients for animals. It is a component of the unusual amino acids selenocysteine and selenomethionine. In humans, selenium is a trace elements nutrient which functions as cofactor for redction of antioxidant enzymes such as glutathione peroxidases and certain forms of thioredoxin reductase found in animals and some plants. Lithium is a highly reactive soft alkali metal with single valence electron. The active principle in this salt is the lithium ion (Li^+).

From the point of inhibition of rice bran lipase, the effect of selenium and lithium ions on the structure-function of RBL were studied. Inactivation of RBL by selenium and lithium ions is expected to yield useful information on the structure-function relationship of lipase and possibly a better method for inactivation of RBL.

The effect of different concentrations of selenium and lithium on the activity of RBL was determined and both selenium and lithium found to decrease the lipase activity. In case of selenium, the enzyme loses nearly 78% of activity at 1×10^{-3} M selenium dioxide concentration and is totally inactivated above 1×10^{-2} M (Fig. 31A). In the case of lithium 72% of the enzyme activity was lost at 1×10^{-3} M concentration of lithium sulfate as shown in Fig. 31B. The RBL loses nearly 18% of activity in 1×10^{-3} M zinc chloride, 22% in 1×10^{-3} M copper sulfate, 19% in 1×10^{-3} M ferric chloride as can be seen from the results only lithium and selenium shows maximum inhibitory effect at lower concentration. In order to confirm the inhibitory effect of lithium, sodium sulfate was used to eliminate the effect of sulfate ions, which did not show any inhibition on the enzyme. Selenium dioxide is one of the most frequently encountered compounds of selenium. Selenium dioxide is a one-dimensional polymer, the chain consisting of alternating selenium and oxygen atoms. Each selenium atom, which is pyramidal, bears a terminal oxide group. The relative stereochemistry at selenium alternates along the polymer chain, this make the selenium highly reactive. Compared to sodium selenite, selenium dioxides are highly reactive. The inhibitory activity profile indicated that inactivation is directly proportional to the concentration of these metal ions, and found that selenium shows more inhibitory effect compared to lithium.

The sulphydryl groups are the functional group of the amino acid cysteine; the thiol group plays an important role in biological systems. Selenium compounds efficiently react with thiols resulting in depletion of thiols, leading to breakdown in proteins tertiary structure. There are a number of reports on selenite and other selenium compounds have been shown to inhibit the functions of Na-K-ATPase and rat brain prostaglandin D synthase by reacting with critical sulfhydryl groups of the proteins, and inhibiting large number sulfhydryl containing enzymes. In fact, the catalytic reaction of selenite with thiols results in a rapid formation of selenide anion, which by redox cycling with oxygen may cause a non stoichiometric oxidation of thiols leading to enzyme inhibition (Fakhrul *et al.,* 1991; Farina *et al.,* 2003). Earlier results of studies also reveals that phenyl boronic acid (PBA), polyphenols such as chlorogenic acid and caffeic acid, which are a potential inhibitor of RBL, was a concentration-dependent inhibition and the inactivation followed a pseudo-first-order kinetics (Raghavendra and Prakash, 2002; Raghavendra *et al.,* 2007).

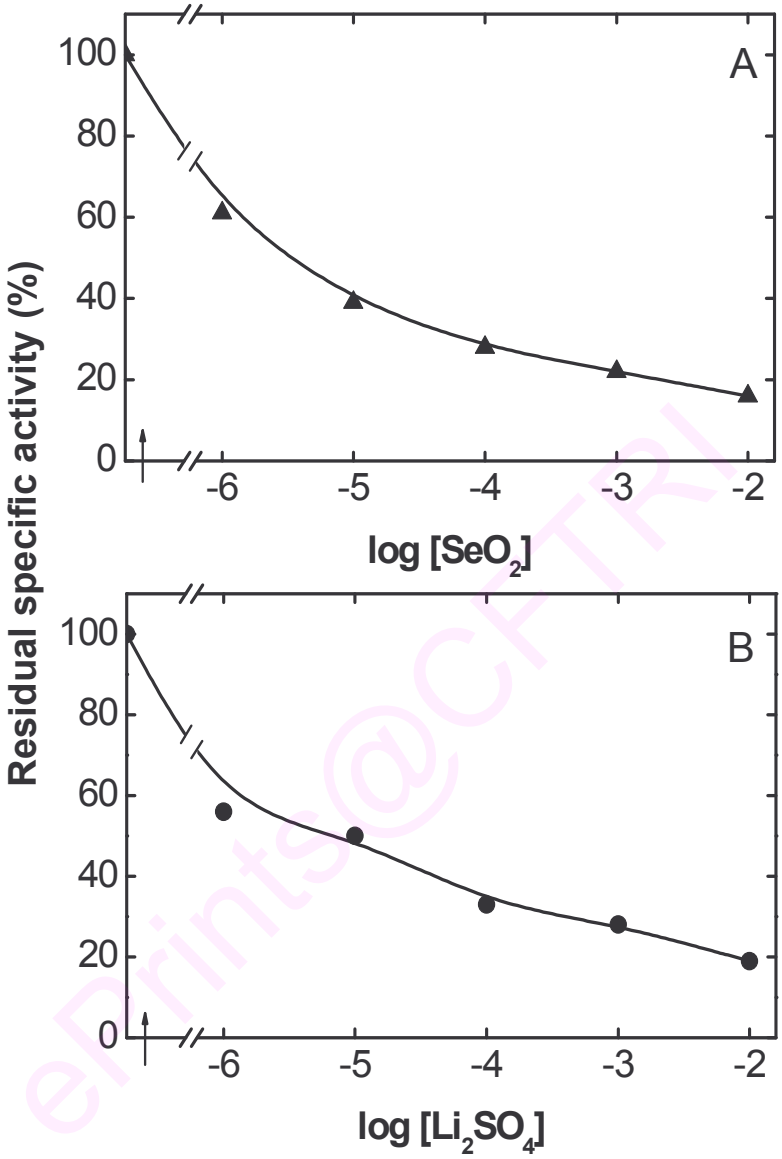

Fig. 31: Effect of SeO_2 (A) and Li_2SO_4 (B) on the activity profile of rice bran lipase. The reaction mixture in 0.05 M sodium phosphate buffer, pH 7.4 was incubated with varying concentration of SeO_2 and Li_2SO_4 at 30°C for 20 min and assayed for remaining activity.

The result of Lineweaver-Burk (or) Double reciprocal plot of initial reaction velocity versus the specified variable substrate concentration at a series of fixed concentrations of selenium dioxide and lithium sulfate is shown in Fig. 32A & B. The velocity of the reaction increases with the substrate concentration thereby indicating that the enzyme show more affinity towards the substrate and it is related to Michaelis constant K_m. The K_m values increases from 3.4 in the absence of selenium dioxide to 5, 7.6, 10 and 20 in the presence of 1×10^{-6} M to 1×10^{-3} M of selenium dioxide respectively. In case of lithium sulfate also the same pattern was observed and the values increases from 3.4 to 4, 7.5, 12.5 and 14 respectively.

The increased value of K_m indicates that the affinity of substrate for the enzyme decreased with increasing concentration of selenium and lithium. The V_{max} values for selenium dioxide and lithium sulfate treated enzyme found to be 20 and 17 respectively. These kinetic analyses suggest that the inhibitor has competitively inhibited the enzyme activity. The K_i value was calculated from the direct linear plot of the slope vs. inhibitor concentration (I°) which was found to be 4×10^{-6} M for lithium sulfate and 1×10^{-6} M for selenium dioxide at pH 7.4. Our earlier inhibition studies of RBL with phenylboronic acid, chlorogenic acid and caffeic acid (Raghavendra and Prakash, 2002; Raghavendra *et al.,* 2007) was also followed the same pattern, where all the above inhibitors acting as a potent in nature with competitive type of inhibition.

The effect of lithium and selenium on the RBL has been checked by size exclusion chromatography on a Superdex-75 column using FPLC (Fig. 33). The native enzyme in buffer alone, selenium (1×10^{-5} M) and lithium (1×10^{-5} M) treated RBL were all eluted with an elution volume of 11.5 ml. From Fig. 33, it can be observed that the intensity of the peaks (b and c) decreases marginally, which represent lithium and selenium treated RBL, respectively as compared to the control peak (a). These data clearly indicate that there are no aggregates and the enzyme has the same exclusion size in the presence of selenium and lithium as indicated by the chromatographic pattern and supports the concept that the RBL was structurally intact in the presence of metal ions.

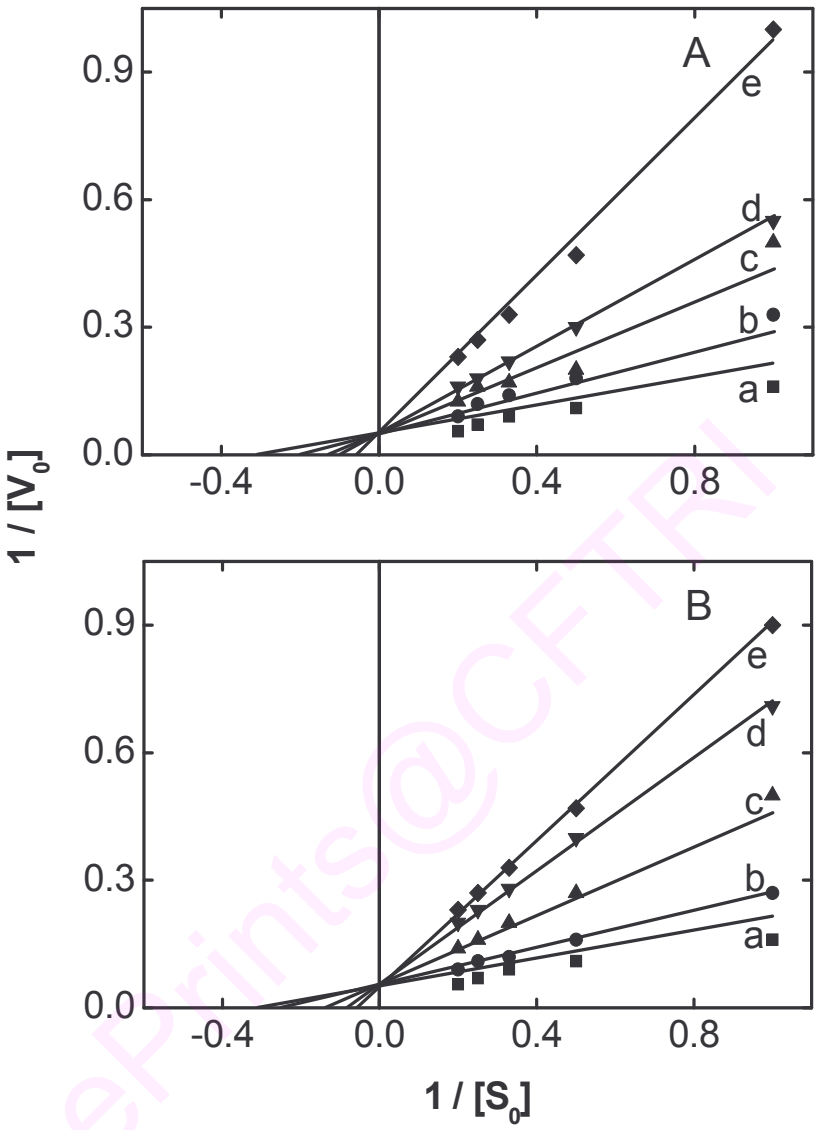

Fig. 32: Lineweaver-Burk plot for the inhibition of rice bran lipase in the presence of different concentrations of inhibitors in 0.05 M sodium phosphate buffer, pH 7.4. (a) control in buffer (0.05 M sodium phosphate buffer, pH 7.4), (b) in 1×10^{-6} M, (c) in 1×10^{-5} M, (d) in 1×10^{-4} M and (e) in 1×10^{-3} M inhibitors. (A) in SeO_2 and (B) in Li_2SO_4.

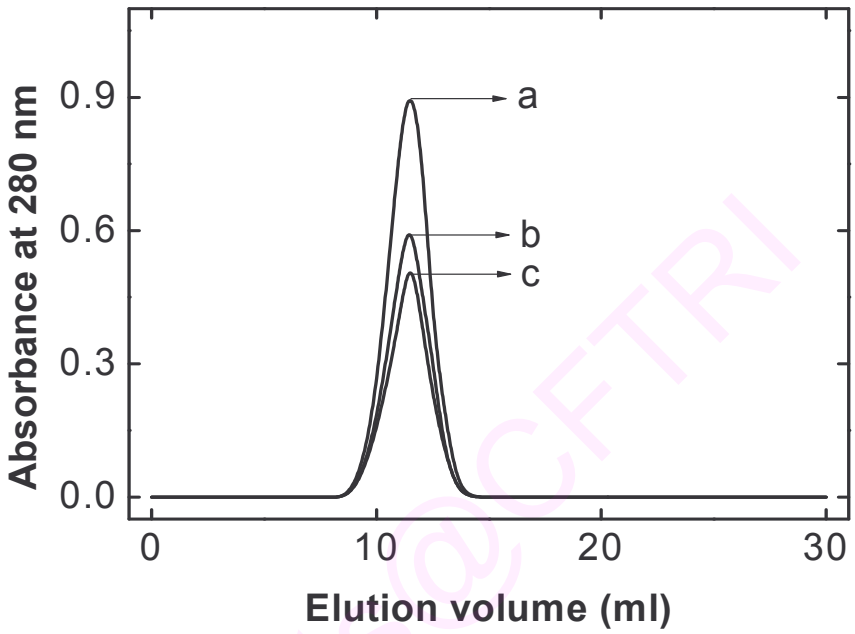

Fig. 33: Elution profile of rice bran lipase (a) in the absence and in the presence of (b) SeO_2 (1×10^{-5} M) and (c) Li_2SO_4 (1×10^{-5} M) in 0.05 M sodium phosphate buffer, pH 7.4.

The effect of these metal ions on the structure of the enzyme was followed by fluorescence and circular dichroic spectroscopic measurements. The secondary structure of rice bran lipase was monitored as a function of selenium dioxide and lithium sulfate by far UV-CD spectroscopy and is shown in Fig. 34. The native protein exhibits a trough at 208 nm and a shoulder at 218 nm. From the Fig. 34, it is clear that the binding of selenium and lithium resulted in a very small change in the CD spectra of RBL, the α-helical content changed from 15% to 14% and 16%, in the absence and presence of 1×10^{-3} M of selenium dioxide and lithium sulfate with minor changes in β-structural content (Table 11).

Fluorescence spectroscopy was carried out to probe the perturbation of tryptophan residue in RBL as a result of selenium dioxide and lithium sulfate interaction with the enzymes and is shown in Fig. 35A & B. RBL has an emission maximum of 337 nm when excited at 285 nm and the fluorescence of RBL is mainly due to the presence of 3 tryptophan residues present in the enzyme. Upon interaction of RBL with selenium dioxide, as shown in Figure 35A, the fluorescence emission maximum of the enzyme shifts to 2 nm with a red shift compared to control with decrease in intensity. In case of lithium sulfate also the spectra followed the same pattern (Fig. 35B). The decrease in the emission intensity and significant red shift in the emission maximum may be attributed to the perturbation of the microenvironment of the tryptophan residues in the presence of these ions. It thus supports the occurrence of the partial unfolding of the enzyme in the presence of these ions.

The above results infer that rice bran lipase activity decreased in presence of selenium dioxide and lithium sulfate. The lipase activity was completely inhibited in presence of selenium dioxide and lithium sulfate above the concentration of 1×10^{-4} M. The mechanism of inhibition might be redox reaction of enzyme with selenium and lithium. Selenium classified as a chalcogen has exquisite metallic properties that can undergo facile redox reaction with sulfur containing amino acids and selenium compounds have well documented inhibitory effect, the mode of inhibition is competitive and active site directed inhibition.

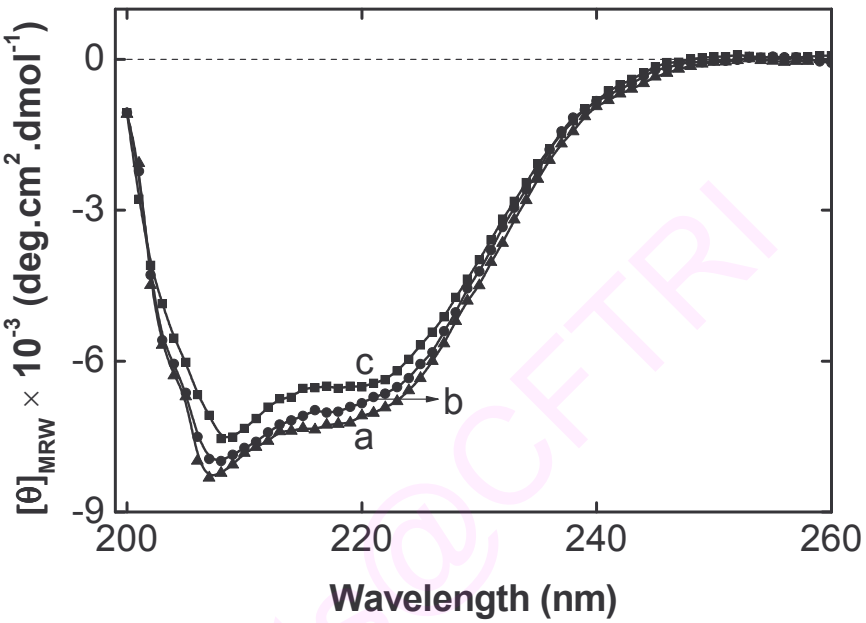

Fig. 34: Far UV-CD spectra of rice bran lipase in the presence of different concentrations of inhibitors in 0.05 M sodium phosphate buffer, pH 7.4 at 25°C. (a) control in buffer (0.05 M sodium phosphate buffer, pH 7.4), (b) in 1×10^{-3} M Li_2SO_4 and (c) in 1×10^{-3} M SeO_2.

Table 11: Secondary structure of rice bran lipase in absence and presence of SeO_2 and Li_2SO_4 in 0.05 M sodium phosphate buffer, pH 7.4.

Secondary structure (%)			
Inhibitor concentration (1×10^{-3} M)	**α-helix**	**β-structure**	**Aperiodic**
Control*	15 ± 1	51 ± 3	34 ± 2
SeO_2	14 ± 1	48 ± 3	38 ± 2
Li_2SO_4	16 ± 1	55 ± 3	29 ± 2

* Native rice bran lipase in 0.05 M sodium phosphate buffer, pH 7.4.

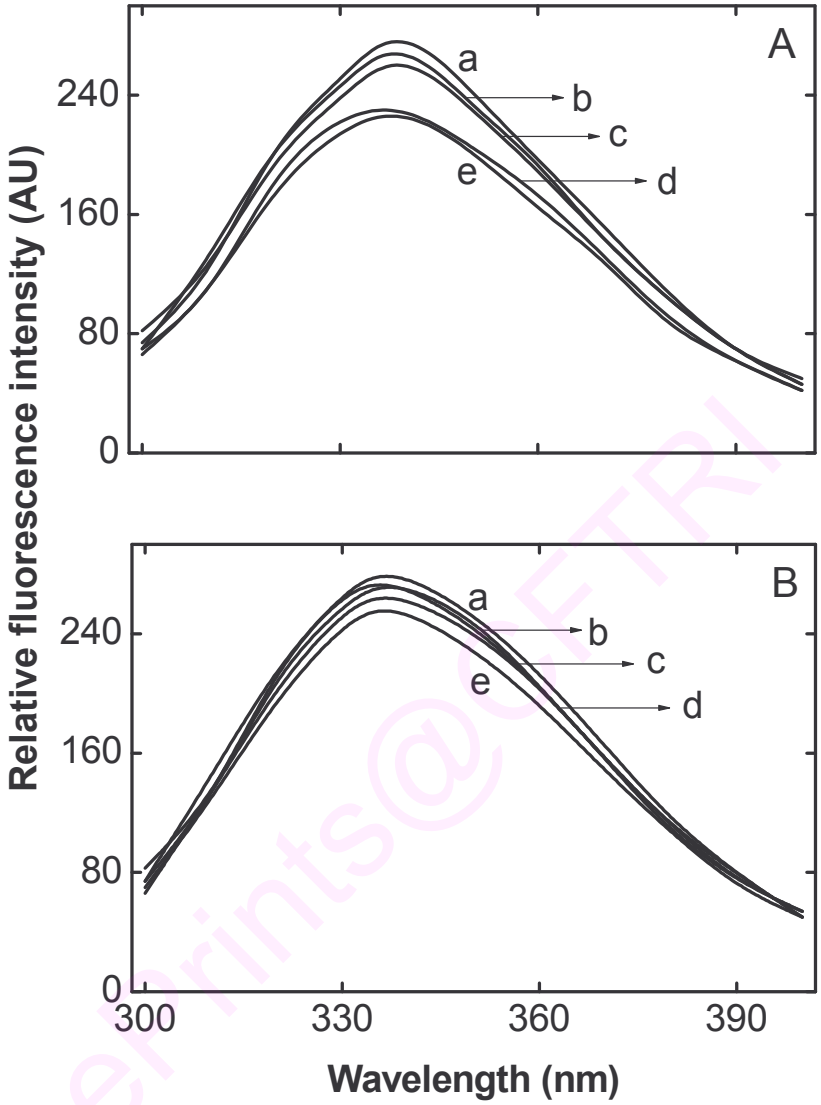

Fig. 35: Intrinsic fluorescence emission spectra of rice bran lipase in the presence of different concentrations of inhibitors in 0.05 M sodium phosphate buffer, pH 7.4. Emission spectra recorded over a range of 300-400 nm. (a) control in buffer (0.05 M sodium phosphate buffer, pH 7.4), (b) in 1×10^{-6} M, (c) in 1×10^{-5} M, (d) in 1×10^{-4} M and (d) in 1×10^{-3} M inhibitors. (A) in SeO_2 and (B) in Li_2SO_4.

Monovalent cations such as lithium inhibit rice bran lipase activity by distorting the geometry of the active site or by retarding product release. From the prospective of kinetics, RBL complexes with lithium lose its ability to carry out catalysis, i.e. it forms a dead-end catalysis. In case of porcine liver fructose 1, 6-bisphosphatase from the kinetic and crystal structure evidence, lithium binds to Glu or Asp residues in the enzyme and these amino acids are very essential for activity (Villeret *et al.,* 1995; Zhang *et al.,* 1996). In case of lipoprotein lipase, lithium inhibited enzyme activity up to 42% by interacting with essential SH group in the active site of the enzyme. Activity of thermophilic Geobacillus sp. TW1 lipase is reduced by about 24% in presence of LiCl (Li and Zhang, 2005).

Thus, the decrease in the activity observed in rice bran lipase was mainly due to the action of selenium and lithium which are known to inhibit the activity of other enzyme by modifying the sulfur containing amino acid and Glu or Asp residues which are mainly essential for catalytic activity of rice bran lipase. Since the active site of the enzyme apparently interacts with these ions which is based on the charge and size. In case of lithium which is monovalent containing one positive charge as compared to selenium atom which as outer four electronic shells, this electronic shell structure gives selenium the versatility to readily donate electron and make the selenium atom an ideal catalytic center. Lipase containing catalytic triad of Ser-His-Asp in its catalytic site and any modification to this triad leads to decrease in its activity. Because both selenium and lithium are known to act on SH containing amino acid (Farina *et al.,* 2003; Villeret *et al.,* 1995; Zhang *et al.,* 1996).

Hence the potential applications of metal ions as an inhibitor of RBL activity eventually prove interesting in terms of physiological and industrial importance. The above data indicates that selenium and lithium inhibits RBL activity, and the nature of inhibition is competitive with no significant change in the protein conformation. These inactivation phenomena of RBL by metal ions provide very useful information for prevention of lipolysis occurring in rice bran, and this method of metal ions treatment will have an advantage compared to physical and chemical treatments in the case of rice bran oil.

3a. 2. Effect of azadirachtin on rice bran lipase activity

3a. 2. Effect of azadirachtin on rice bran lipase inhibitory activity

The neem tree (*Azadirachta indica*), has been increasingly attracting the interest of researchers from various fields. More than 145 compounds have been isolated and characterized; these properties are due to a large number of secondary plant metabolites found in various parts of the tree (Johnson and Morgan, 1997; Kumar *et al.,* 2003). Azadirachtin, a tetranotriterpenoid is remarkable both for its chemical complexity and for its biological activity.

Considerable progress has been achieved regarding the biological activity and medicinal application of azadirachtin. In case of alkaline phosphatase and adenosine triphosphatase the activity decreases in presence of azadirachtin (Senthil Nathan *et al.,* 2005) lactate dehydrogenase an important glycolytic enzyme present in all animal tissue and involved in carbohydrate metabolism looses its activity drastically in presence of azadirachtin (Senthil Nathan *et al.,* 2006). But their spectrum and level of action on other enzymes are not studied. Recent finding from our laboratory suggest pronounced effect of neem seed extract on stabilization of rice bran (Ganagdhara and Prakash, 2005) by reducing the free fatty acid level in the neem seed extract treated bran by about 70% compared to the control. Thus the role of azadirachtin on the inhibition of rice bran lipase was investigated for its inhibitory effect.

In order to understand the mode of interaction of azadrirachtin on rice bran lipase the mechanism of interaction is studied by activity, kinetic and structural studies. Interaction of rice bran lipase (RBL) by azadirachtin is expected to yield useful information on the structure-function relationship of rice bran lipase with an ultimate goal of exploiting the potential of plant source for producing edible grade oil from rice bran.

The effect of azadirachtin from neem seed on rice bran stability was measured by the change in total FFA is shown in Fig. 36. The FFA level in raw rice bran increased rapidly from an initial value of 2.5 to 16% during 120 hours of storage respectively, when stored at room temperature (25°C). In contrast, the FFA level reached 3.6% in azadirachtin treated bran, when stored at room temperature (25°C).

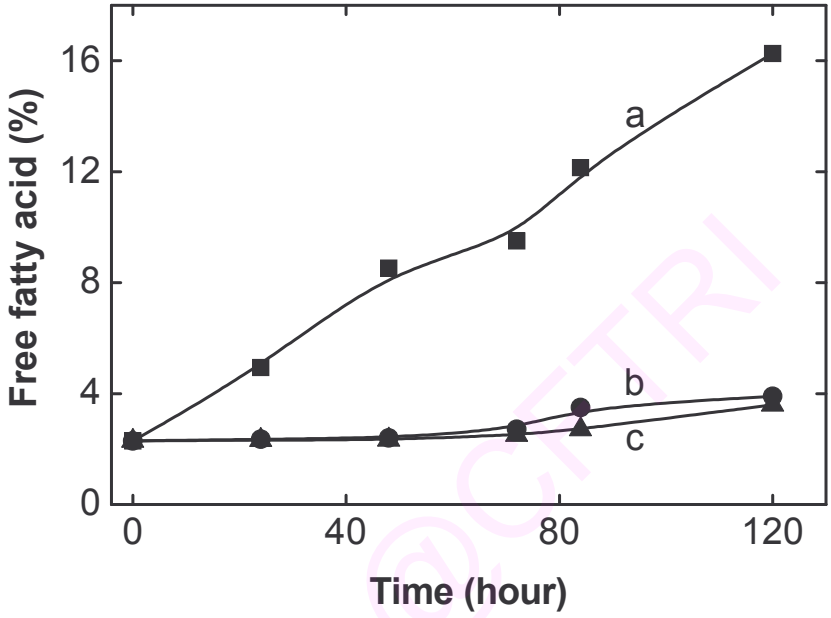

Fig. 36: Effect of azadirachtin from neem seed extract on free fatty acid formation on rice bran. (a) fresh rice bran (b) rice bran treated with azadirachtin from neem seed extract in the ratio of 1:5 (c) rice bran treated with azadirachtin from neem seed extract in the ratio of 1:7.

The increase in FFA content of raw rice bran in this study is similar to the results obtained by other researchers (Lam and Proctor, 2002; Champagne *et al.,* 1992). This is result of the rapid development of hydrolytic rancidity in raw rice bran, which makes this product unsuitable for human consumption. The addition of azadirachtin at 1×10^{-3} M concentration, evidenced by decrease in free fatty acid content from 16% to 3.6% after 120 hours. Thus, the decrease in the free fatty acid content observed was mainly due to the effect of azadirachtin on lipase.

The extent of inactivation of enzyme in the presence of azadirachtin was monitored by activity measurement in different concentrations of azadirachtin. In Figure 37 is shown the activity profile of the enzyme in the presence of azadirachtin. From the Figure, it can be seen that the enzyme activity decreases linearly as the concentration of the azadirachtin increases. The loss was found to be 84% at 1 mM azadirachtin concentration. The activity results clearly demonstrate that the RBL inhibition is more pronounced at higher concentration of azadirachtin. The mechanism of inhibition mainly involves interaction of azadirachtin with one of the amino acid in the catalytic triad which is very important for lipase activity such as aspartic acid. From the interaction studies Pravin Kumar *et al.,* (2007) shown that there is charge based interaction between azadirachtin and Arg, Lys, Glu, Asp, this in turn leads to loss in enzyme activity.

The effect of substrate on RBL inactivation by azadirachtin is depicted by means of the Lineweaver-Burk (or) double reciprocal plot wherein the initial reaction velocity is compared with the specified variable substrate concentrations at a series of fixed inhibitor (Fig. 38). The velocity of the reaction increases with the increase in the concentrations of the substrate, depicting the affinity of enzyme toward substrate and it is related to the Michaelis constant. The K_m value increase from 3.4 in the absence of the inhibitor to 5.2, 7.7, 11, 17 and 33 in presence of 0.2 to 1 mM azadirachtin concentration respectively. The increasing values of K_m indicate that the affinity of the substrate for the enzyme decreased with increasing concentration of azadirachtin. The K_i value was calculated from the direct linear plot of the slope versus inhibitor concentration and found to be 0.14 mM. This indicates the alteration of surface parameters of the enzyme by inhibitors. V_{max} values was unchanged in the presence of

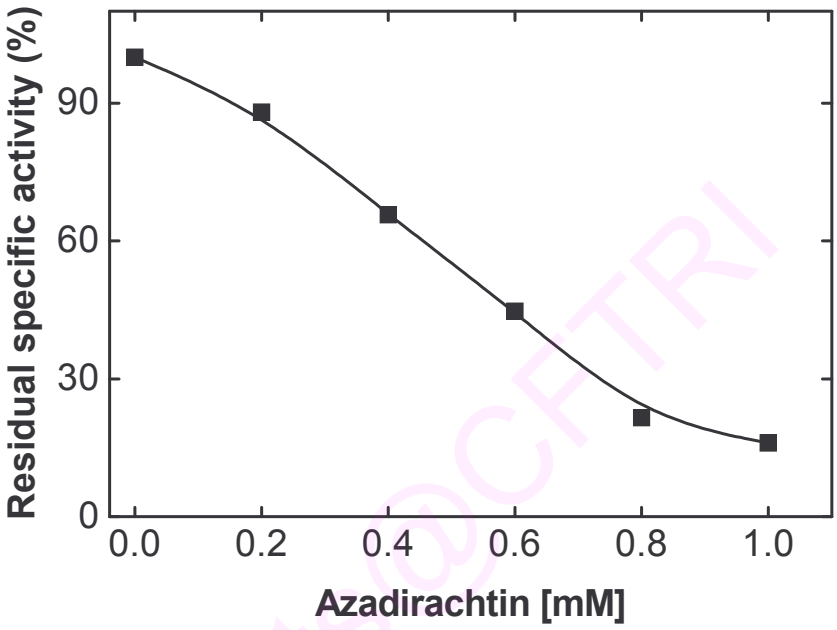

Fig. 37: Effect of azadirachtin on the activity profile of rice bran lipase. The reaction mixture in 0.05 M sodium phosphate buffer, pH 7.4 was incubated with varying concentration of azadirachtin at 30°C for 20 min and assayed for remaining activity.

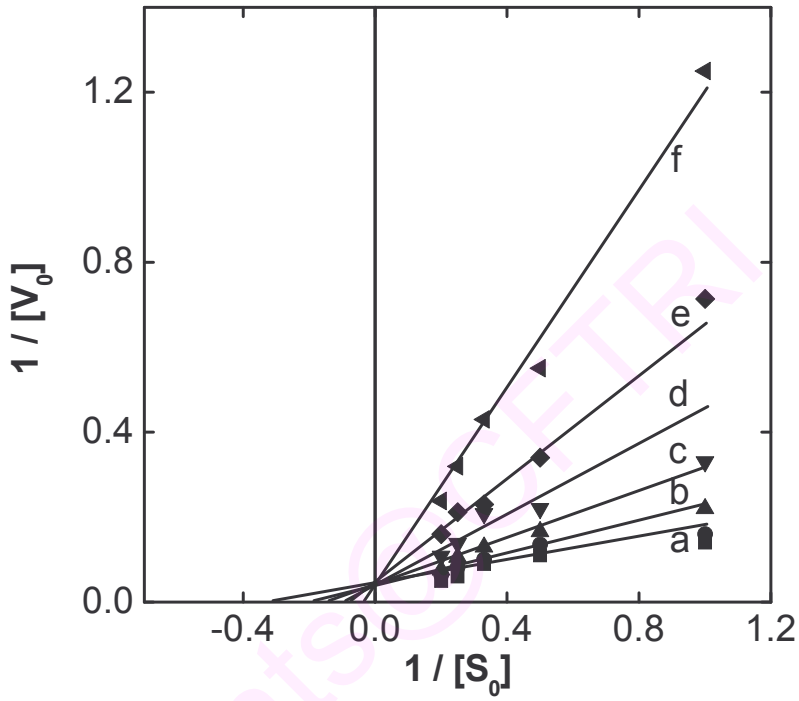

Fig. 38: Lineweaver-Burk plots for the inhibition of rice bran lipase in the presence of different concentrations of azadirachtin in 0.05 M sodium phosphate buffer, pH 7.4. (a) control in buffer (0.05 M sodium phosphate buffer, pH 7.4), (b) in 0.2 mM, (c) in 0.4 mM, (d) in 0.6 mM, (e) in 0.8 mM and (f) in 1.0 mM azadirachtin.

the inhibitors, and this clearly proves that affinity of the inhibitor is more effective towards the lipase rather than substrate and further supported by the values of inhibitor constant (K_i).

Thus, kinetic analysis suggests that the nature of inhibition was competitive; that means both the substrate and the inhibitor compete for the same active site. He, (2003) has shown that esterase activity was competitively inhibited by azadirachtin. Azadirachtin is shown to bind aspartate, which is a part of catalytic triad in lipase (Pravin Kumar *et al.,* 2007). It directly binds to the lipase active site therefore, reduces the concentration of free enzyme available for catalytic reaction. The reaction between azadirachtin and rice bran lipase is reversible. After removal of azadirachtin from lipase by gel filtration enzyme regains its activity. This clearly indicates that azadirachtin is not covalently bond to the enzyme.

The effect of azadirachtin on the stability of the RBL has been checked by size exclusion chromatography on a Superdex-75 column using FPLC (Fig. 39). The native enzyme in buffer alone and azadirachtin (0.8 mM) treated RBL were all eluted with an elution volume of 11.5 ml. From Figure 39, it can be observed that the intensity of the peak (b) decreases marginally, which represent azadirachtin treated RBL as compared to the control peak (a). These data clearly indicate that there are no aggregates and the enzyme has the same exclusion size in the presence of azadirachtin as indicated by the chromatographic pattern and supports the concept that the RBL was structurally intact in the presence of azadirachtin.

To understand the structural changes upon azadirachtin addition, the secondary structural features of RBL are evaluated in the presence and the absence of azadirachtin by following the far UV-CD spectral measurements. The studies shown that there was a minor change in the secondary structure of RBL as a result of azadirachtin addition and the spectra are shown in Fig. 40. The enzyme exhibits 2 well-defined minima around 208 and 220 nm. The azadirachtin treated enzyme shown to decrease in the ellipticity from 206 to 225 nm. From the Figure 40, it is clear that the binding of azadirachtin resulted in a very small change in the CD spectra of RBL by decreasing the α-helical content from 15% to 13% and 11%, in the absence and presence of 0.4 and 0.8 mM azadirachtin with decrease in β-structure (Table 12).

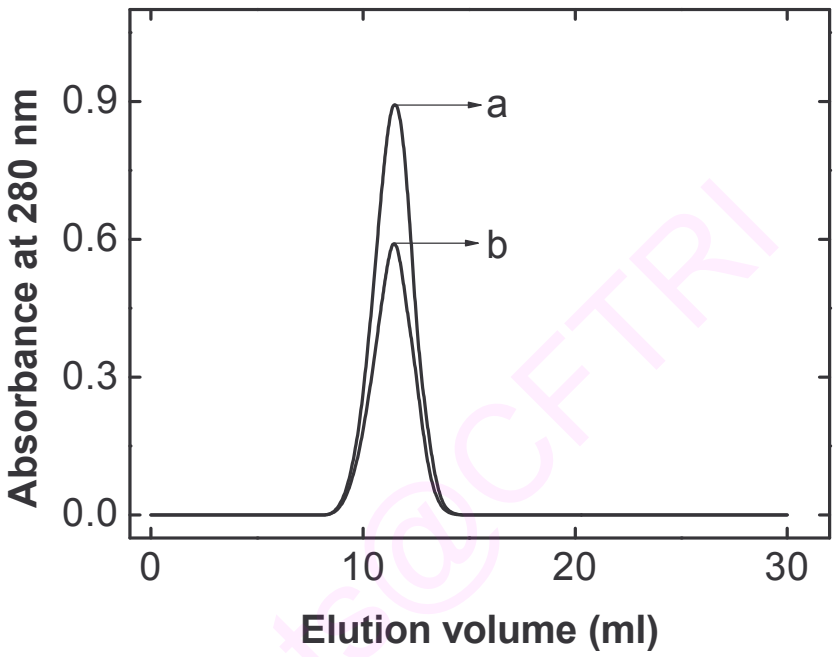

Fig. 39: Elution profile of rice bran lipase (a) in the absence and presence of (b) 0.8 mM of azadirachtin in 0.05 M sodium phosphate buffer, pH 7.4.

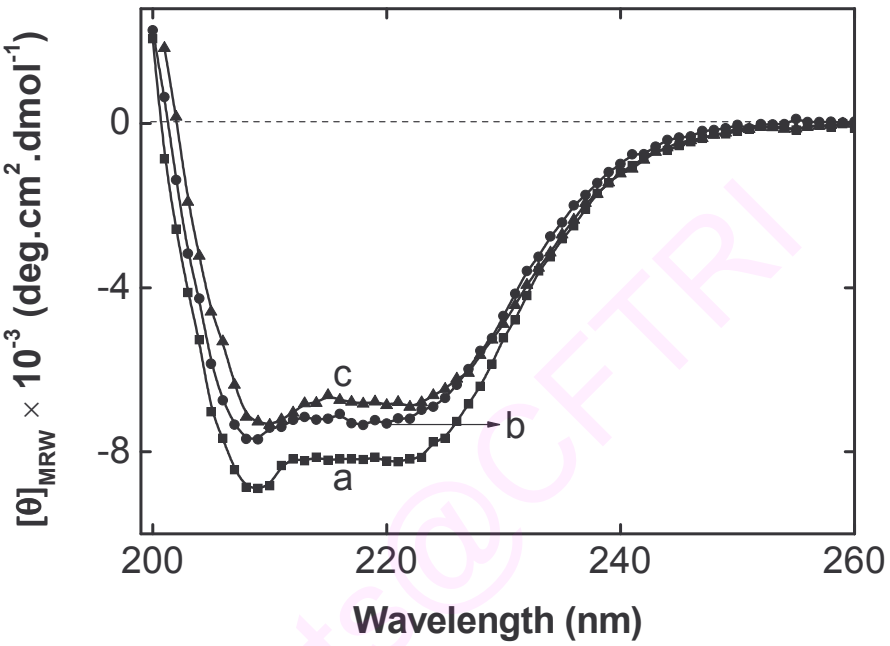

Fig. 40: Far UV-CD spectra of rice bran lipase in the presence of different concentrations of azadirachtin in 0.05 M sodium phosphate buffer, pH 7.4 at 25°C. (a) control in buffer (0.05 M sodium phosphate buffer, pH 7.4), (b) in 0.4 mM and (c) in 0.8 mM azadirachtin.

Table 12: Secondary structure of rice bran lipase in absence and presence of azadirachtin in 0.05 M sodium phosphate buffer, pH 7.4.

Secondary structure (%)			
Azadirachtin concentration (mM)	**α-helix**	**β-structure**	**Aperiodic**
Control*	15 ± 1	51 ± 3	34 ± 2
0.4	13 ± 1	48 ± 3	39 ± 2
0.8	11 ± 1	46 ± 2	43 ± 2

* Native rice bran lipase in 0.05 M sodium phosphate buffer, pH 7.4.

Fluorescence studies were carried out to support the mechanism of inactivation of rice bran lipase in presence of different concentration of azadirachtin. The intrinsic fluorescence spectra in presence of azadirachtin indicate the perturbation of tryptophan residues as shown in Fig. 41. The fluorescence intensity was found to decrease with the increasing concentration of azadirachtin. There was a red shift in the emission maxima attributed to relocalization of tryptophan residues in more polar environment. Based on the structural analyses of several lipases, tryptophan was identified as a constituent of the central part of the 'lid' covering the active site of the enzyme (Brzozowski *et al.,* 1991).

The binding of azadirachtin to the enzyme may cause a change in the environment either at the active site or in a region close to the active site of the enzyme leading to its reduced activity. Studies also indicate that alkaline phosphatase and adenosine triphosphatase activity was inhibited by azadirachtin (Senthil Nathan *et al.,* 2005). Lactate dehydrogenase an important glycolytic enzyme looses its activity drastically in presence of azadirachtin (Senthil Nathan *et al.,* 2006). The main mechanism of bonding is considered to be noncovalent interactions in the case of RBL as indicated in this study from reversibility studies.

The phenolic groups of azadirachtin can form hydrogen bonds with the polar groups (amide, guanidine, peptide, amino, and carboxyl groups) of protein. With the recognition that there are many hydrophobic amino acids present in the enzyme, such as proline, phenylalanine, tyrosine, and tryptophan, it could be considered that azadirachtin would have a higher tendency to bind the enzymes through hydrophobic association. The occurrence of the hydrogen bond and the hydrophobic association will change the enzyme molecular configuration, resulting in an impact on the enzyme activities.

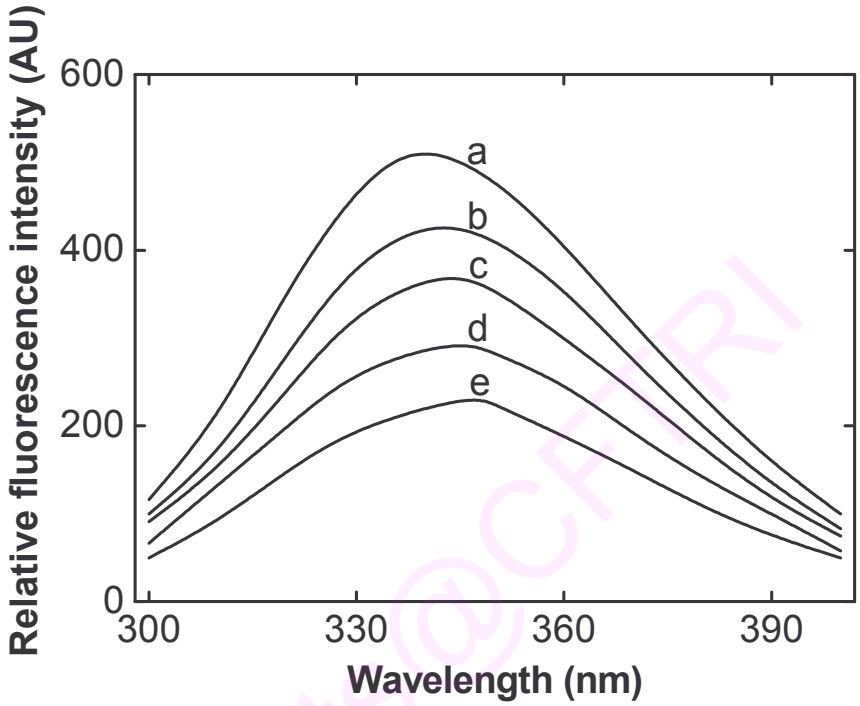

Fig. 41: Intrinsic fluorescence emission spectra of rice bran lipase in the presence of different concentrations of azadirachtin in 0.05 M sodium phosphate buffer, pH 7.4. Emission spectra recorded over a range of 300-400 nm. (a) control in buffer (0.05 M sodium phosphate buffer, pH 7.4), (b) in 0.2 mM, (c) in 0.4 mM, (d) in 0.6 mM and (e) in 0.8 mM azadirachtin.

The above study indicates clearly that azadirachtin inhibits RBL activity. The nature of inhibition was found to be competitive and reversible along with minor conformational changes in the protein structure. This provides useful information for the mechanism of inactivation of rice bran lipase from structural as well as kinetic point of view. The inactivation phenomena of RBL by naturally occurring secondary plant metabolites provide very useful information for prevention of lipolysis occurring in rice bran. This detailed investigation of the inhibitory effect of azadirachtin on RBL leads to the development of the processes toward stabilization of rice bran.

3b. Effect of cosolvents on the activity, stability, structure-function, kinetics and preferential interaction studies of porcine pancreatic lipase

3b. Effect of cosolvents on the activity, stability, structure-function, kinetics and preferential interaction studies of porcine pancreatic lipase

The stability of proteins against changes in the thermodynamic and chemical conditions of the solvent is only marginal. Considerable effort is devoted to improve protein stability, which is the result of a balance between the intramolecular interactions of protein functional groups and their interaction with solvent environment (Timasheff, 1993; Yancey *et al.,* 1982). Protein stability can be studied both from thermodynamic, as well as kinetic approaches (Baker and Agard, 1994; Plaza del Pino *et al.,* 2000).

The mechanism by which cosolvents stabilize the proteins has been extensively studied from thermodynamic and kinetic angles (Arakawa and Timasheff, 1985; Timasheff, 1993; Yancey *et al.,* 1982). Cosolvents increase the chemical potential of a protein via weak interactions (Schellman, 1990). Cosolvents have been used since long for the stabilization of enzymes and proteins. Therefore, the development of the techniques for enhancing the activity and stability of enzymes continues to be a focus of attention.

The effect of cosolvents on rice bran lipase activity has already been worked out; the results clearly demonstrate that rice bran lipase activity stabilizes in different cosolvents. In order to see the similarity of stabization of lipase with cosolvents porcine pancreatic lipase was selected instead of rice bran lipase.

Porcine pancreatic lipase (PPL) is an exocrine enzyme catalyses the hydrolysis of emulsified glycerides (esters of glycerol and fatty acids) at the interface of an aqueous and non-aqueous phase (Aloulou *et al.,* 2006). This is one of the most frequently used enzymes in organic chemistry because of its wide specificity and high regio and enantioselectivity (Reetz, 2002). Lipases may be inactivated by slight changes in the physicochemical parameters such as temperature, pressure, pH and

ionic strength. The aim of this work was to obtain porcine pancreatic lipase with new properties, such as stability and high hydrolytic activity for industrial applications.

In this chapter, activity, stability and mechanism of thermal and pH mediated stability of PPL is examined in presence of selected cosolvents (glycerol, xylitol, sorbitol, sucrose and trehalose). Cosolvents mediated mechanism of stabilization is looked upon using kinetic and thermodynamic approaches. The structural studies were carried out using circular dichroic and fluorescence spectra to estimate the structural alterations upon the interaction of cosolvents with enzymes. The preferential interaction parameter was measured in presence of different concentration of cosolvents to asses the relationship between thermal stability and relative hydration of proteins in presence of cosolvents. Thus, present investigation would ultimately provide the energetics of the cosolvent-enzyme interactions to understand at a molecular level the forces that are responsible for the stability of the PPL in these cosolvents.

3b. 1. Homogeneity of porcine pancreatic lipase enzyme

The homogeneity of the porcine pancreatic lipase was evaluated by SDS-PAGE and isoelectric focusing method by determining its pI. The lipase from porcine pancreas (PPL) is a single polypeptide chain of 50 kDa protein with an isoelectric point of 5.5 (Fig. 42A & B). The lipase assay was carried out by using tributyrin as a substrate at its optimum conditions i.e. at pH 7.5 and 37°C.

3b. 2. Effect of cosolvents on the activity, stability, structure-function, kinetics and preferential interaction studies of PPL

The effect of selected cosolvents such as glycerol, xylitol, sorbitol, sucrose and trehalose on the activity of PPL was monitored; these cosolvents did not show any activation effect on PPL at optimum condition. Thermal stability studies of PPL at a fixed period of time and temperature was measured using above cosolvents with different concentrations with a description of results using activity measurements, kinetic studies, partial specific volume measurements, thermal denaturation measurements, fluorescence spectroscopy and circular dichroic spectroscopy measurements.

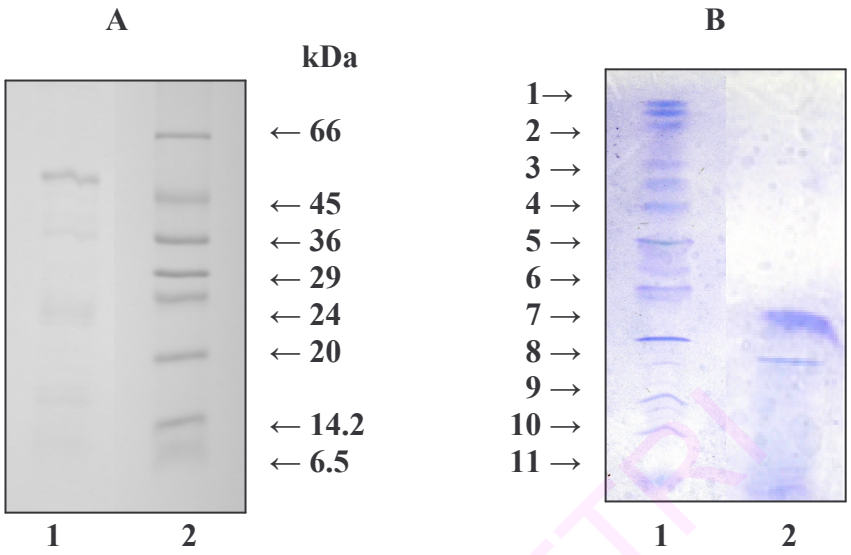

Fig. 42: (A) SDS-PAGE pattern of proteins. (Lane 1) Lipase from porcine pancreas (50 kD). (Lane 2) Standard proteins-BSA (66 kDa), Ovalbumin (45 kDa), Glyceraldyhyde-3-phosphate dehydrogenase (36 kDa), Carbonic anhydrase (29 kDa), Trypsinogen (24 kDa), Trypsin inhibitor (20 kDa), α-Lactalbumin (14.2 kDa) and Aprotinin (6.5 kDa).

(B) Isoelectric focusing pattern of proteins in Ampholine PAG plate pH 3.5-9.5. (Lane 1) Standard pI marker. (1) Trypsinogen (pI 9.30), (2) Lentil lectin-based band (pI 8.65), (3) Lentil lectin-middle band (pI 8.15), (4) Lentil lectin-acidic band (pI 8.15), (5) Myoglobin-basic band (pI 7.35), (6) Myoglobin-acidic band (pI 6.85), (7) Human carbonic anhydrase B (pI 6.55), (8) Bovine carbonic anhydrase B (pI 5.85), (9) β-lactoglobulin A (pI 5.20), (10) Soybean trypsin inhibitor (pI 4.55) and (11) Amyloglucosidase (pI 3.50). (Lane 2) Lipase from porcine pancreas (pI 5.5 ± 0.1).

The activity profile of PPL as a function of temperature has shown that sharp decrease in the activity at higher temperature. PPL activity was monitored after short exposure to higher temperature, i.e., 60°C for 10 min, both in presence and absence of selected cosolvents such as glycerol, xylitol, sorbitol, sucrose and trehalose. Figure 43 shows the effect of cosolvents (glycerol, xylitol, sorbitol sucrose and trehalose) on thermal inactivation profile of lipase. The enzyme loses nearly 97% of its activity, after incubation for 10 min at 60°C. In presence of cosolvents the activity was protected to different extent. The maximum protection was obtained with 40% trehalose which shown a protection of 78% activity. The presence of other cosolvents also shows the same effects with 66, 60, 53 and 21% protection of activity in 40% sorbitol, xylitol, sucrose and 30% glycerol concentration respectively (Table 13).

The above result clearly indicates that, the presence of cosolvents protected the activity of lipase against thermal inactivation. Enzyme activity was also checked for its reversibility of its original activity after removal of cosolvent by gel filtration on Sephadex G-25 column. Enzyme fractions eluted in the void volume were used for reversibility studies. From the reversibility studies it is clear that sorbitol and trehalose at 40% concentration exerts maximum protection against thermal inactivation as evidenced by maximum recovery of residual specific activity values of nearly 20 and 15% respectively. Sucrose and xylitol at 40% concentration shows 12 and 13% recovery of residual specific activity. Glycerol found to be least effective compared to above solvents with a recovery value of only 7% at 30% concentration (Table 13).

The above results clearly indicating that, cosolvents were protecting the enzyme activity by increasing the thermal stability at higher temperature. Protection of activity at higher temperature depends on individual cosolvents and its concentration. Based on the above results the effectiveness of different cosolvents in protecting the enzyme activity is in the order (at 40% concentration).

Trehalose > Sorbitol > Xylitol > Sucrose > Glycerol

Kinetic parameters were measured to evaluate the mode of thermal stability to understand the activity protection, by measuring the initial velocity of PPL catalysis

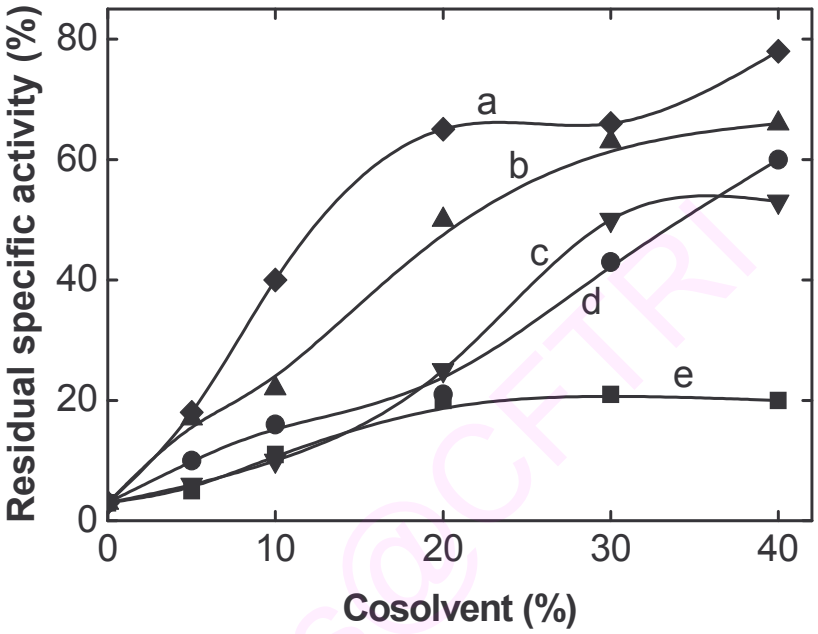

Fig. 43: Thermal inactivation profile of porcine pancreatic lipase in the presence of different concentrations of cosolvents in 0.01 M Tris-HCl buffer, pH 7.5. The reaction mixture was exposed to 60°C for 10 min with and without cosolvents (0-40%). (a) in trehalose, (b) in sorbitol, (c) in sucrose, (d) in xylitol and (e) in glycerol.

Table 13: Residual specific activity of porcine pancreatic lipase as a function of cosolvent concentrations after exposure to 60°C for 10 min.

Cosolvent concentration (%) (w/v)		Residual specific activity (%)*	
		In presence of cosolvent	After removal of cosolvent
Glycerol	0**	3 ± 0.2	0
	10	11 ± 1	2 ± 0.2
	20	20 ± 2	6 ± 0.5
	30	21 ± 2	7 ± 0.5
	40	20 ± 2	6 ± 0.5
Xylitol	0**	3 ± 0.2	0
	10	16 ± 1	6 ± 0.5
	20	21 ± 2	8 ± 1
	30	43 ± 3	11 ± 2
	40	60 ± 4	13 ± 2
Sorbitol	0**	3 ± 0.2	0
	10	22 ± 2	10 ± 1
	20	50 ± 3	12 ± 2
	30	63 ± 4	14 ± 2
	40	66 ± 4	20 ± 3
Sucrose	0**	3 ± 0.2	0
	10	10 ± 1	4 ± 0.3
	20	25 ± 2	8 ± 1
	30	50 ± 3	9 ± 1
	40	53 ± 4	12 ± 2
Trehalose	0**	3 ± 0.2	0
	10	40 ± 3	8 ± 1
	20	65 ± 4	11 ± 1
	30	66 ± 4	13 ± 2
	40	78 ± 5	15 ± 2

* Heat treated PPL (60°C for 10 min) in 0.01 M Tris-HCl buffer, pH 7.5.

** Controls were run parallely in all these experiments.

in presence of different cosolvents, after incubating the enzyme at 60°C for 10 min. The kinetic parameters were determined by reciprocal plot of initial velocity and substrate concentration. As shown in Table 14, the Michalies constant (K_m) values decreases in presence of cosolvents compared to control. In presence of 30% glycerol, 40% xylitol, sorbitol, sucrose and trehalose K_m value were found to be 12, 9, 8, 11 and 7 mM respectively compared to the control value of 19 mM. The catalytic constant (k_{cat}) was increased in presence of cosolvents at each concentration. Thorough analysis of all these results reveals that lower K_m values with increase in k_{cat} values results in a higher catalytic efficiency of cosolvent treated enzyme.

In order to study the nature of interactions between cosolvents and protein the preferential interaction parameters were determined in presence and absence of different concentrations of cosolvents. The preferential interaction parameters were measured by estimating the partial specific volumes of proteins in presence of cosolvents at isomolal and isopotential conditions. The isomolal and isopotential conditions refer the identical molality and chemical potential of cosolvents in protein solutions.

The partial specific volume of PPL was measured as a function of different protein concentration (8-25 mg/ml) in 0.01 M Tris-HCl buffer, pH 7.5 at 20°C. The partial specific volumes of PPL were determined to be 0.728 ml/g and 0.729 ml/g in isomolal and isopotential conditions respectively. Representative plots of apparent partial specific volume against protein concentration of PPL with different concentration of cosolvents are represented (Fig. 44 & 45). In all cases, there was no protein concentration dependence of the apparent partial specific volume in the systems with or without cosolvents at conditions of constant isomolal and isopotential condition. In all cases, however, there was a good linear relationship between the apparent partial specific volume and protein concentration.

From the partial specific volume measurements, the preferential interaction parameters of PPL in presence of different cosolvents (glycerol, xylitol, sorbitol, sucrose and trehalose) ranging the concentration between 0-40% by weight/volume at 20°C were calculated.

Table 14: Kinetic parameters of porcine pancreatic lipase in absence and presence of different concentrations of cosolvents after exposure to 60°C for 10 min.

Cosolvent concentration (%) (w/v)	k_{cat} (10^3 min^{-1})	K_m (mM)	k_{cat}/K_m (10^3 min^{-1} mM^{-1})
Control (native)*	194 ± 8	5 ± 0.5	38.8
Heat treated (in buffer)**	3 ± 0.5	19 ± 2	0.16
30% glycerol	41 ± 3	12 ± 2	3.40
40% xylitol	116 ± 6	9 ± 1	12.9
40% sorbitol	128 ± 7	8 ± 1	16.0
40% sucrose	101 ± 7	11 ± 2	9.20
40% trehalose	152 ± 9	7 ± 1	21.7

* Native PPL in 0.01 M Tris-HCl buffer, pH 7.5.

** Heat treated PPL in 0.01 M Tris-HCl buffer, pH 7.5.

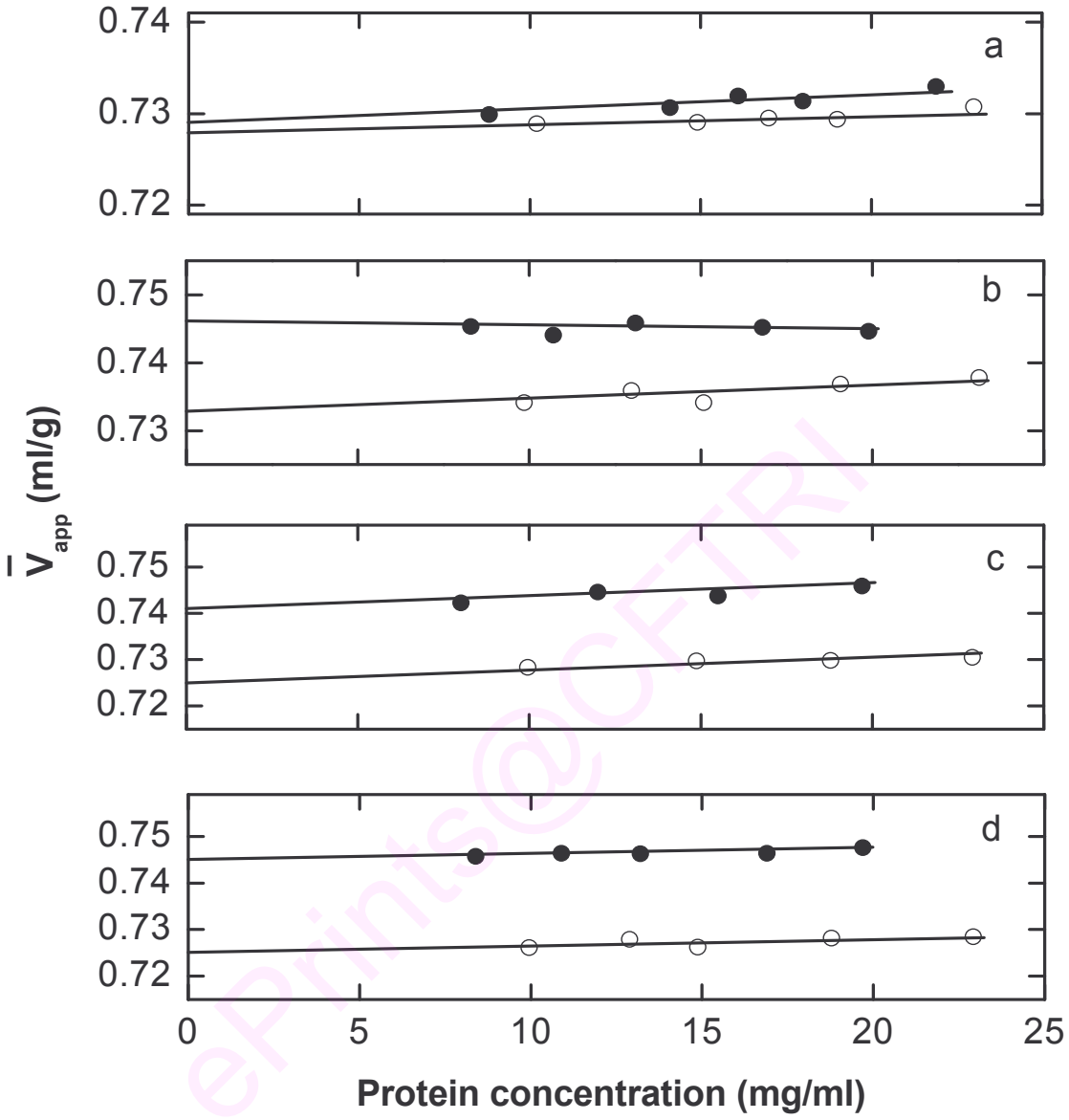

Fig. 44: Apparent partial specific volume of porcine pancreatic lipase in the presence of different concentrations of cosolvents in 0.01 M Tris-HCl buffer, pH 7.5 under isomolal (○) and isopotential (●) conditions at 20°C. (a) control in buffer (0.01 M Tris-HCl buffer, pH 7.5), (b) in 40% sorbitol, (c) in 30% glycerol and (d) in 30% xylitol.

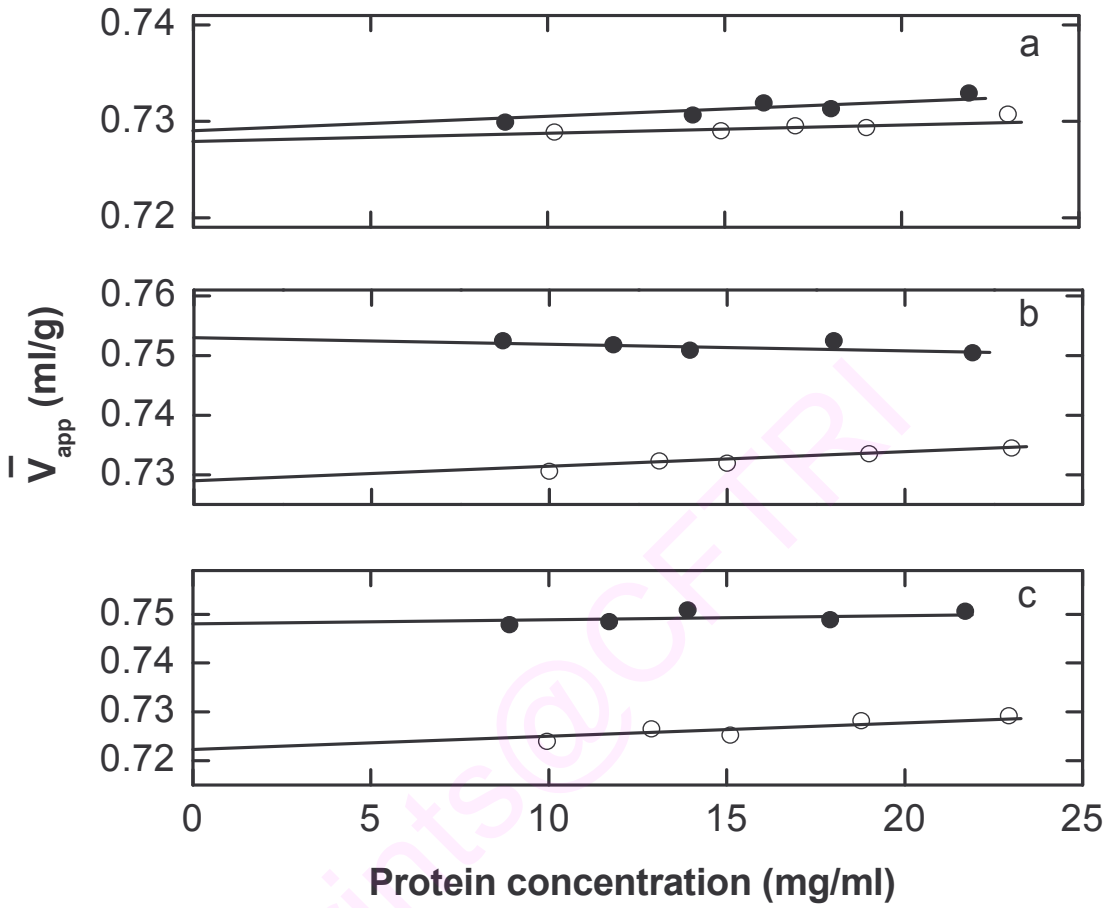

Fig. 45: Apparent partial specific volume of porcine pancreatic lipase in the presence of different concentrations of cosolvents in 0.01 M Tris-HCl buffer, pH 7.5 under isomolal (○) and isopotential (●) conditions at 20°C. (a) control in buffer (0.01 M Tris-HCl buffer, pH 7.5), (b) in 40% sucrose and (c) in 30% trehalose.

From Table 15, the values of the partial specific volume of PPL in glycerol at concentration of 10, 20, 30 and 40% by w/v were 0.726, 0.726, 0.725 and 0.724 ml/g for isomolal and 0.731, 0.735, 0.741 and 0.738 ml/g for isopotential conditions. With all the concentration of glycerol isopotential value was found to increase with increasing glycerol concentration and conversely, with slight decrease in isomolal value. The partial specific volume of PPL in xylitol are shown in Table 16, at a concentration of 10, 20, 30 and 40% by w/v were 0.728, 0.727, 0.725 and 0.728 ml/g for isomolal and 0.734, 0.739, 0.745 and 0.745 ml/g for isopotential conditions. In all the concentrations of xylitol, increase in isopotential value with increase in concentration up to 30% then it becomes constant, conversely there was little change in the isomolal values. With sorbitol the values were found to be 0.730, 0.731, 0.732 and 0.733 ml/g for isomolal and 0.737, 0.740, 0.745 and 0.746 ml/g for isopotential condition (Table 17).

In presence of sorbitol is obtained an increase in the isomolal value of $\bar{v}$ with increase in concentration of the cosolvent. In presence of sucrose the isomolal values were, 0.728, 0.724, 0.723 and 0.722 ml/g with isopotential values of 0.740, 0.745, 0.750 and 0.748 ml/g for 10-40% concentration (Table 18). In presence of trehalose the isomolal values were 0.729, 0.726, 0.729 and 0.730 ml/g with isopotential values of 0.739, 0.746, 0.753 and 0.750 ml/g for 10-40% concentration (Table 19). From the isomolal and isopotential values, preferential interaction or binding parameters of PPL in cosolvents were calculated. Preferential interaction parameter for glycerol, xylitol, sorbitol, sucrose and trehalose on g/g basis are shown in Fig. 46.

In case of sorbitol, sucrose and trehalose the preferential hydration parameter decreases linearly with cosolvents concentrations. The values of preferential interaction parameter values found to be maximum in 30% glycerol, 30% xylitol, 40% sorbitol, 40% sucrose and 30% trehalose concentration with a value of −0.083, −0.070, −0.046, −0.079 and −0.076 g/g and the lowest of −0.019 g/g in 10% xylitol respectively. The interaction parameter on a mol/mol basis was also calculated, showing highest value of −45.2 in the case of 30% glycerol and the lowest of −4.6 in 10% sucrose concentration.

Table 15: Apparent partial specific volume and preferential interaction parameter values of porcine pancreatic lipase as a function of glycerol.

Interaction parameters	Glycerol (%) (w/v)			
	10	**20**	**30**	**40**
ϕ_2^0 (ml/g)	0.726 ± 0.001	0.726 ± 0.001	0.725 ± 0.001	0.724 ± 0.001
$\phi`_2^0$ (ml/g)	0.731 ± 0.001	0.735 ± 0.002	0.741 ± 0.002	0.738 ± 0.002
g_3 (g/g)	0.108	0.236	0.390	0.578
m_3 (mol of solvent per 1000 g H_2O)	1.17	2.57	4.24	6.28
$(\delta g_3/\delta g_2)T,\mu_1,\mu_3$ (g/g)	−0.022 ± 0.007	−0.042 ± 0.014	−0.083 ± 0.016	−0.080 ± 0.016
$(\delta g_1/\delta g_2)T,\mu_1,\mu_3$ (g/g)	0.202 ± 0.030	0.180 ± 0.025	0.214 ± 0.034	0.138 ± 0.022
$(\delta m_3/\delta m_2)T,\mu_1,\mu_3$ (mol/mol)	−11.8 ± 2.2	−23.1 ± 4.6	−45.2 ± 5.2	−43.3 ± 6.2

Table 16: Apparent partial specific volume and preferential interaction parameter values of porcine pancreatic lipase as a function of xylitol.

Interaction parameters	Xylitol (%) (w/v)			
	10	**20**	**30**	**40**
ϕ_2^0 (ml/g)	0.728 ± 0.001	0.727 ± 0.001	0.725 ± 0.001	0.728 ± 0.001
$\phi`_2^0$ (ml/g)	0.734 ± 0.001	0.739 ± 0.002	0.745 ± 0.002	0.745 ± 0.002
g_3 (g/g)	0.107	0.231	0.377	0.549
m_3 (mol of solvent per 1000 g H_2O)	0.70	1.52	2.47	3.61
$(\delta g_3/\delta g_2)T,\mu_1,\mu_3$ (g/g)	−0.019 ± 0.003	−0.039 ± 0.005	−0.070 ± 0.012	−0.064 ± 0.009
$(\delta g_1/\delta g_2)T,\mu_1,\mu_3$ (g/g)	0.174 ± 0.012	0.170 ± 0.009	0.186 ± 0.014	0.116 ± 0.007
$(\delta m_3/\delta m_2)T,\mu_1,\mu_3$ (mol/mol)	−6.15 ± 1.2	−12.9 ± 1.8	−23.0 ± 3.1	−20.9 ± 2.5

Table 17: Apparent partial specific volume and preferential interaction parameter values of porcine pancreatic lipase as a function of sorbitol.

Interaction parameters	Sorbitol (%) (w/v)			
	10	20	30	40
ϕ_2^0 (ml/g)	0.730 ± 0.001	0.731 ± 0.001	0.732 ± 0.001	0.733 ± 0.001
$\phi`_2^0$ (ml/g)	0.737 ± 0.002	0.740 ± 0.002	0.745 ± 0.002	0.746 ± 0.002
g_3 (g/g)	0.107	0.230	0.374	0.544
m_3 (mol of solvent per 1000 g H_2O)	0.59	1.26	2.05	2.99
$(\delta g_3/\delta g_2)T,\mu_1,\mu_3$ (g/g)	−0.021 ± 0.002	−0.028 ± 0.003	−0.043 ± 0.004	−0.046 ± 0.004
$(\delta g_1/\delta g_2)T,\mu_1,\mu_3$ (g/g)	0.194 ± 0.014	0.121 ± 0.006	0.114 ± 0.005	0.084 ± 0.003
$(\delta m_3/\delta m_2)T,\mu_1,\mu_3$ (mol/mol)	−5.70 ± 0.8	−7.65 ± 0.9	−11.7 ± 1.2	−12.5 ± 1.4

Table 18: Apparent partial specific volume and preferential interaction parameter values of porcine pancreatic lipase as a function of sucrose.

Interaction parameters	Sucrose (%) (w/v)			
	10	20	30	40
ϕ_2^0 (ml/g)	0.728 ± 0.001	0.724 ± 0.001	0.723 ± 0.002	0.722 ± 0.002
$\phi`_2^0$ (ml/g)	0.740 ± 0.001	0.745 ± 0.002	0.750 ± 0.002	0.748 ± 0.002
g_3 (g/g)	0.107	0.228	0.368	0.533
m_3 (mol of solvent per 1000 g H_2O)	0.31	0.66	1.07	1.55
$(\delta g_3/\delta g_2)T,\mu_1,\mu_3$ (g/g)	−0.032 ± 0.003	−0.058 ± 0.006	−0.078 ± 0.007	−0.079 ± 0.007
$(\delta g_1/\delta g_2)T,\mu_1,\mu_3$ (g/g)	0.298 ± 0.021	0.253 ± 0.019	0.211 ± 0.012	0.148 ± 0.009
$(\delta m_3/\delta m_2)T,\mu_1,\mu_3$ (mol/mol)	−4.64 ± 0.3	−8.43 ± 1.5	−11.4 ± 1.8	−11.5 ± 2.0

Table 19: Apparent partial specific volume and preferential interaction parameter values of porcine pancreatic lipase as a function of trehalose.

Interaction parameters	Trehalose (%) (w/v)			
	10	**20**	**30**	**40**
ϕ_2^0 (ml/g)	0.729 ± 0.001	0.726 ± 0.001	0.729 ± 0.001	0.730 ± 0.001
$\phi`_2^0$ (ml/g)	0.739 ± 0.001	0.746 ± 0.002	0.753 ± 0.002	0.750 ± 0.002
g_3 (g/g)	0.107	0.229	0.372	0.541
m_3 (mol of solvent per 1000 g H_2O)	0.31	0.67	1.08	1.58
$(\delta g_3/\delta g_2)T,\mu_1,\mu_3$ (g/g)	−0.028 ± 0.004	−0.060 ± 0.006	−0.076 ± 0.007	−0.067 ± 0.006
$(\delta g_1/\delta g_2)T,\mu_1,\mu_3$ (g/g)	0.267 ± 0.019	0.261 ± 0.017	0.204 ± 0.013	0.124 ± 0.008
$(\delta m_3/\delta m_2)T,\mu_1,\mu_3$ (mol/mol)	−4.18 ± 0.25	−8.75 ± 1.6	−11.10 ± 1.9	−9.83 ± 1.8

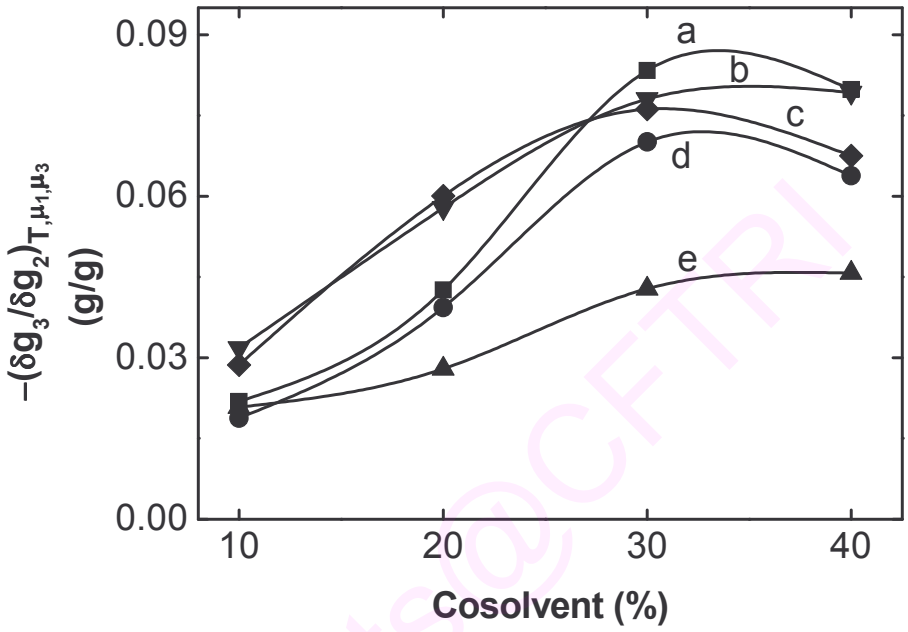

Fig. 46: Effect of cosolvents on the preferential interaction parameter of porcine pancreatic lipase on g/g basis in the concentration range of 0-40%, w/v. The values were calculated from the isomolal and isopotential partial specific volume measurements. (a) in glycerol, (b) in sucrose, (c) in trehalose, (d) in xylitol and (e) in sorbitol.

From measurements of the preferential interactions of PPL with cosolvent components, it is evident that organic solute to stabilize the structure of protein is related to preferential hydration of the protein in an aqueous solution containing these cosolvents. Preferential hydration is a thermodynamic phenomenon that reflects the inability of cosolvents to interact with protein molecule; thus, it leads to an exclusion of these cosolvent components from the protein domain. A number of studies by Timasheff and co-workers on different cosolvent system such as glycerol (Gekko and Timasheff, 1981a), sucrose (Lee and Timasheff, 1981), sorbitol (Xie and Timasheff, 1997a), trehalose (Xie and Timasheff, 1997b), and xylitol (Gekko and Morikawa, 1981a), have explained the phenomenon of preferential hydration.

In Figure 47A & B, is show the far UV-CD spectra of PPL in presence of various concentrations of cosolvents at 60°C. From the Figure it is clear that heat treated enzyme looses its secondary structure. On the other hand, in presence of various concentrations of cosolvents the molar ellipticity values are decreased with more ordered structure. At higher temperature the structure of PPL is extensively denatured, there is retention of the secondary structure in presence of cosolvent solutions relative to that in the buffer alone (Table 20). A similar observation has been made earlier (Gekko and Morikawa, 1981b; Kaushik and Bhat, 2003; Lee and Timasheff, 1981).

The intrinsic fluorescence spectra of PPL at higher temperature are an excellent parameter to monitor the polarity of tryptophan environment, and are sensitive to the protein conformation. Fluorescence spectra were monitored after PPL was exposed to 60°C for 10 min in presence and absence of cosolvents (Fig. 48A-C & 49A-B). Incubation at 60°C for 10 min, leads to a shift in the emission maximum from 343 to 350 nm with a decrease in fluorescence intensity. This indicates alteration in the polarity of aromatic amino acid residues. Melo *et al.,* 2000, observed the same effect at higher temperature in case of *Chromobacterium viscosum* lipase.

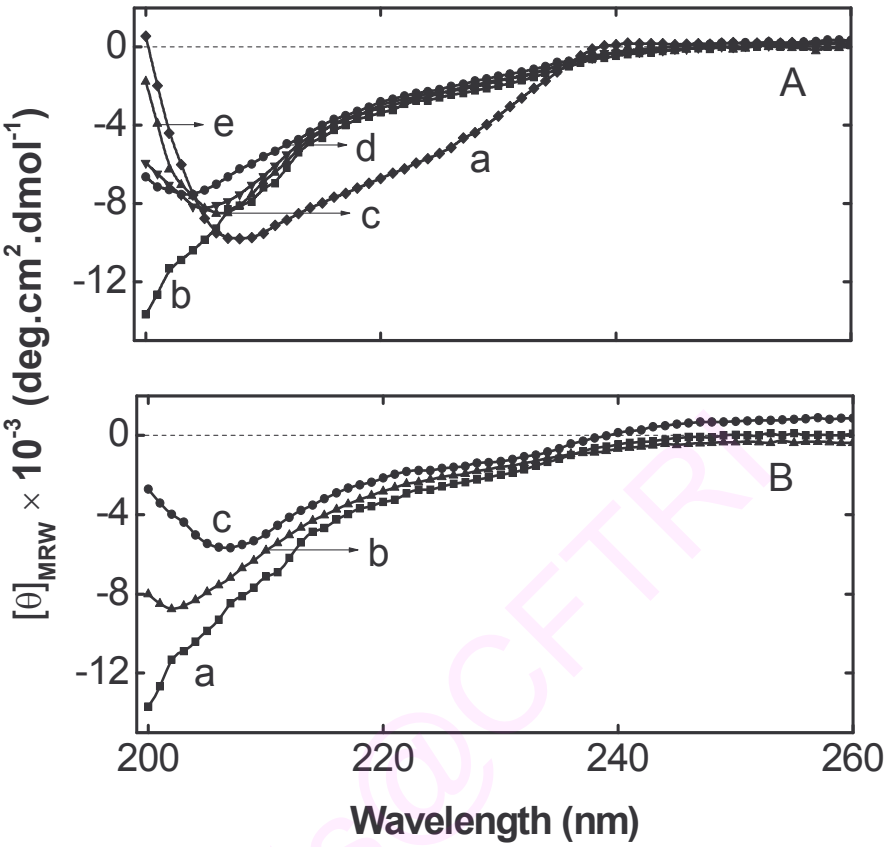

Fig. 47: (A) Far UV-CD spectra of porcine pancreatic lipase in the presence of different concentrations of cosolvents in 0.01 M Tris-HCl buffer, pH 7.5 after exposure to 60°C for 10 min. (a) control in native condition without heat treatment (0.01 M Tris-HCl buffer, pH 7.5), (b) control heat treated (without any cosolvent), (c) in 30% sorbitol, (d) in 30% glycerol and (e) in 30% xylitol.

(B) Far UV-CD spectra of porcine pancreatic lipase in the presence of different concentrations of cosolvents in 0.01 M Tris-HCl buffer, pH 7.5 after exposure to 60°C for 10 min. (a) control heat treated (without any cosolvent), (b) in 30% sucrose and (c) in 30% trehalose.

Table 20: Secondary structure of porcine pancreatic lipase in absence and presence of different cosolvents after exposure to 60°C for 10 min.

Secondary structure (%)			
Cosolvent concentration (%) (w/v)	**α-helix**	**β-structure**	**Aperiodic**
Control (native)*	23 ± 1	24 ± 2	53 ± 3
Heat treated (in buffer)**	3 ± 1	25 ± 2	72 ± 3
30% glycerol	12 ± 1	29 ± 2	59 ± 3
30% xylitol	11 ± 1	25 ± 2	64 ± 3
30% sorbitol	15 ± 1	26 ± 2	59 ± 3
30% sucrose	12 ± 1	26 ± 2	62 ± 3
30% trehalose	10 ± 1	26 ± 2	64 ± 3

* Native PPL in 0.01 M Tris-HCl buffer, pH 7.5.

** Heat treated PPL after exposure to 60°C for 10 min in 0.01 M Tris-HCl buffer, pH 7.5.

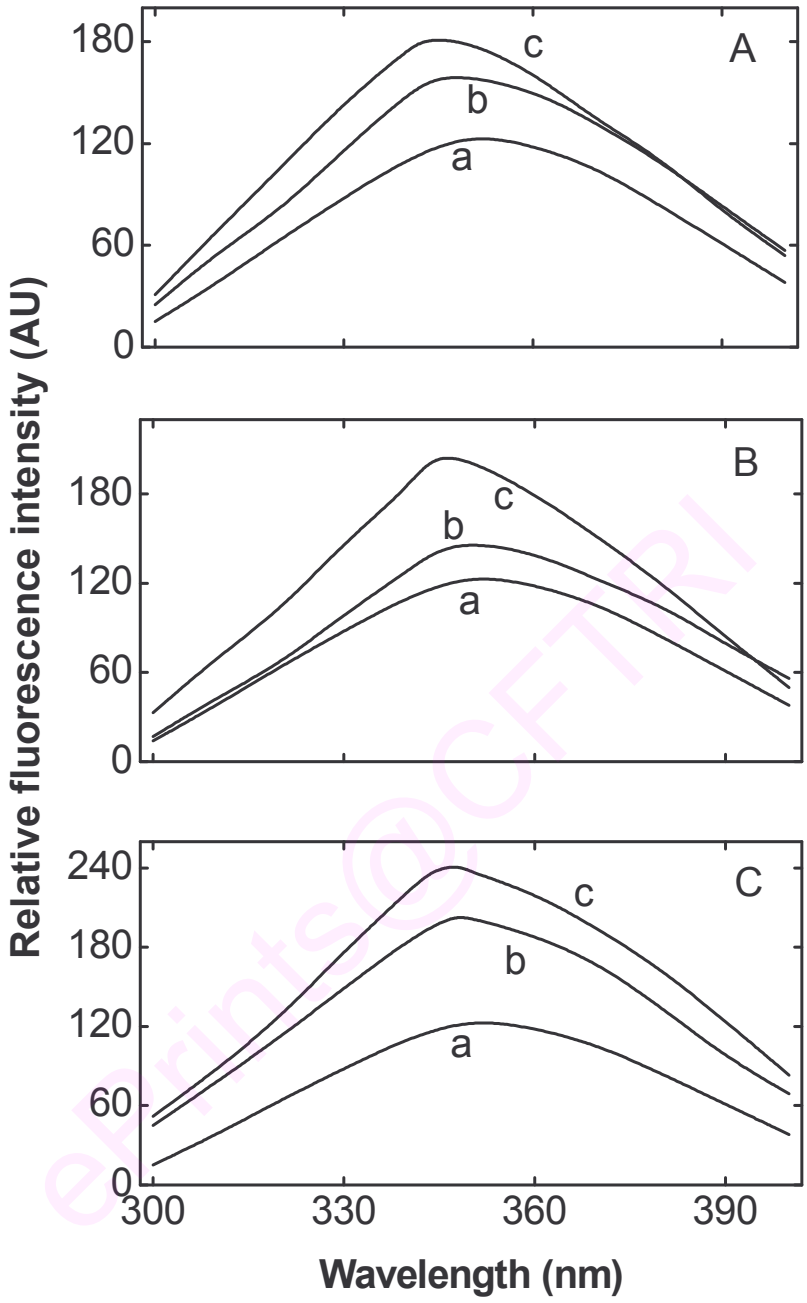

Fig. 48: Intrinsic fluorescence emission spectra of porcine pancreatic lipase in the presence of different concentrations of cosolvents in 0.01 M Tris-HCl buffer, pH 7.5 after exposure to 60°C for 10 min. Emission spectra recorded over a range of 300-400 nm. (a) control in buffer (0.01 M Tris-HCl buffer, pH 7.5), (b) in 20% and (c) in 30% cosolvents. (A) in glycerol, (B) in xylitol and (C) in sorbitol.

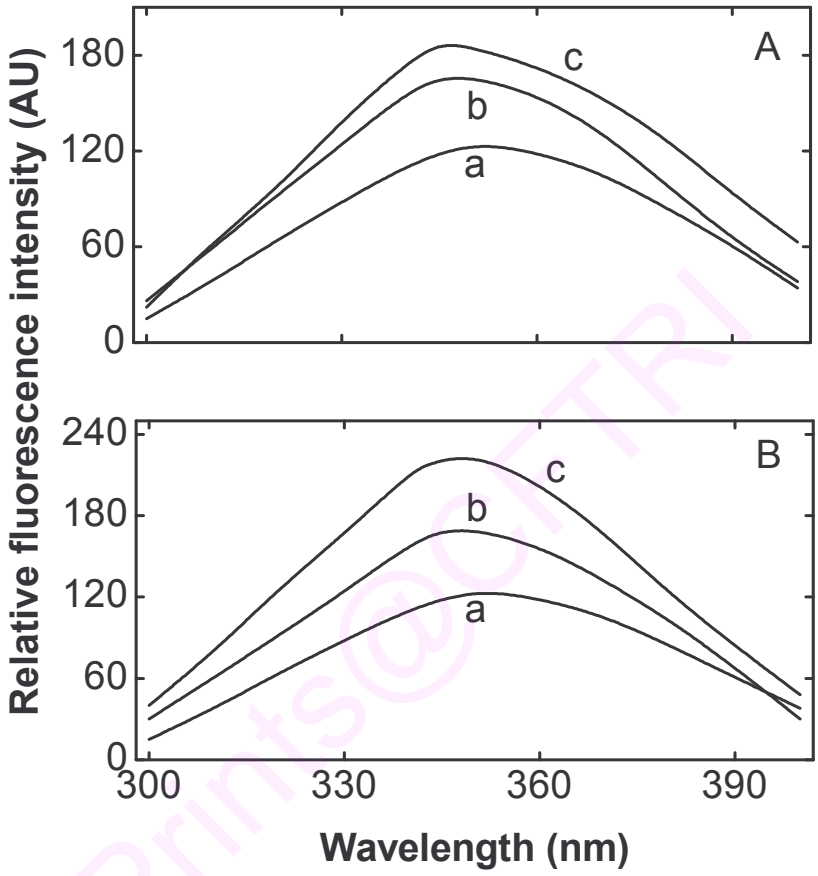

Fig. 49: Intrinsic fluorescence emission spectra of porcine pancreatic lipase in the presence of different concentrations of cosolvents in 0.01 M Tris-HCl buffer, pH 7.5 after exposure to 60°C for 10 min. Emission spectra recorded over a range of 300-400 nm. (a) control in buffer (0.01 M Tris-HCl buffer, pH 7.5), (b) in 20% and (c) in 30% cosolvents. (A) in sucrose and (B) in trehalose.

In presence of all cosolvents blue shift observed with increase in fluorescence intensity, leads to a shift in the emission maximum from 350 to a minimum of 343 nm with increase in fluorescence intensity at various concentrations of cosolvents. It is clear that addition of cosolvents causes an elevation of the thermal transition temperature of PPL. Taking the midpoint of the transition as the thermal denaturation temperature (T_m), it can be seen that the thermal denaturation temperature increased monotonely with different cosolvent concentrations. The thermal transition curves of PPL at various cosolvent concentrations are shown in Fig. 50A-C & 51A-B. The increase being greater at 40% trehalose, the denaturation temperature was raised from a control value of 48°C to a value of 66°C. The apparent T_m value in presence of 10, 20, 30 and 40% trehalose the T_m was 54, 59, 61 and 66°C, respectively. In presence of 10, 20, 30 and 40% sorbitol the T_m was 55, 59, 62 and 64°C, respectively. Xylitol shows the protection of maximum up to 14°C. The apparent T_m increased to 52, 55, 61 and 64°C at 10, 20, 30 and 40% concentration respectively. Compared to above cosolvents sucrose and glycerol were found to less effective, in presence of sucrose the apparent T_m value increased to 52, 55, 59 and 63°C at 10, 20, 30 and 40% concentration. Glycerol found to be least effective compared to all solvents apparent T_m value increased to 52, 57, 59 and 62°C at 10, 20, 30 and 40% concentration respectively.

The increase in the apparent T_m of PPL may be attributed to the nature of the cosolvents used and the different kinds of effects they exert such as preferential exclusion, solvophobic interaction between the peptide backbone and osmolytes, surface tension effect (Baier and McClements, 2005; Kaushik and Bhat, 2003; Xie and Timasheff, 1997c).

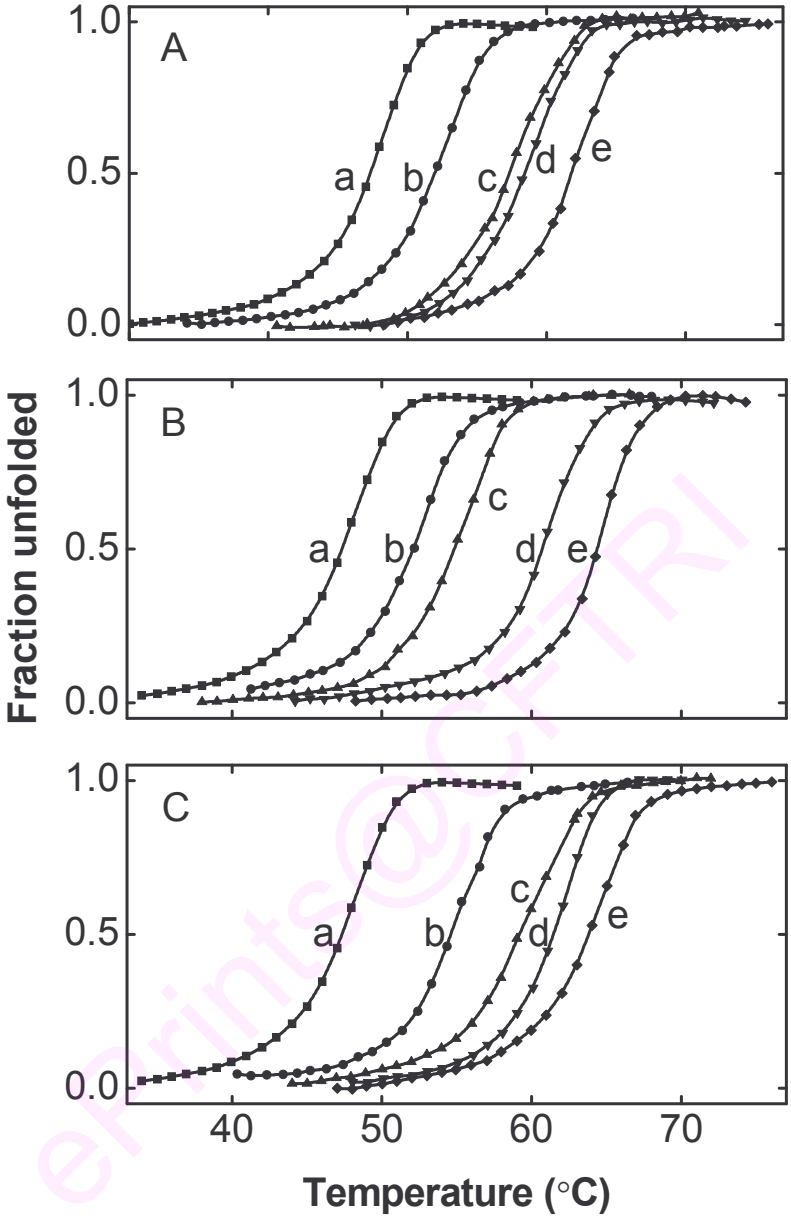

Fig. 50: Apparent thermal denaturation curves of porcine pancreatic lipase in the presence of different concentrations of cosolvents in 0.01 M Tris-HCl buffer, pH 7.5. The absorption spectra were recorded as a function of temperature at 287 nm. (a) control in buffer (0.01 M Tris-HCl buffer, pH 7.5), (b) in 10%, (c) in 20%, (d) in 30% and (e) in 40% cosolvents. (A) in glycerol, (B) in xylitol and (C) in sorbitol.

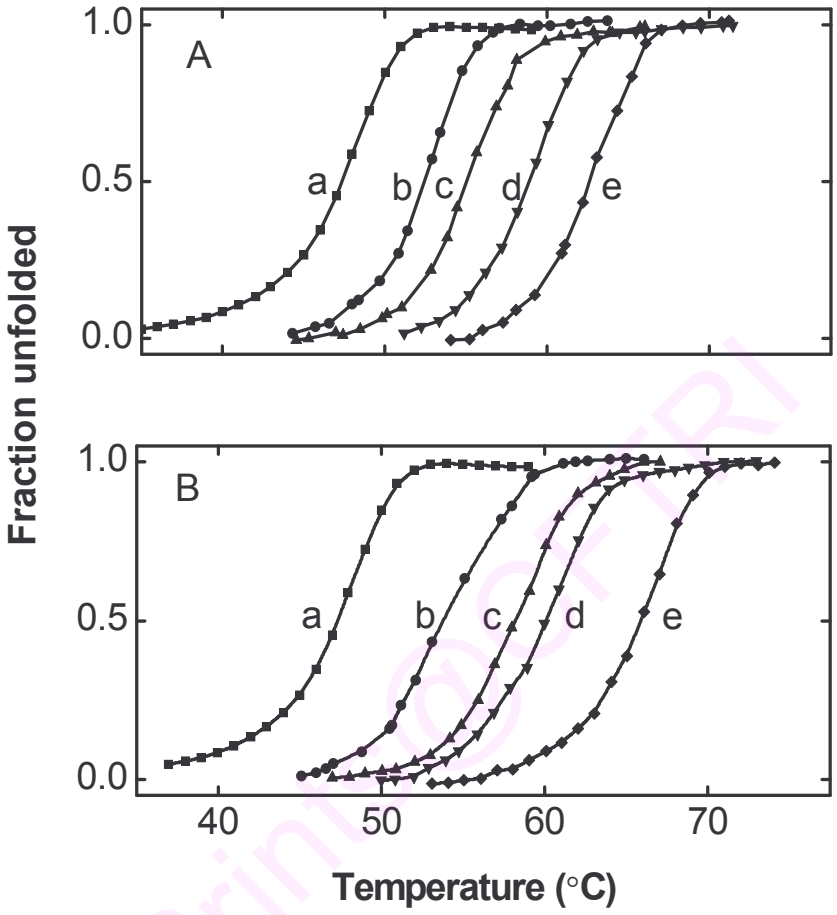

Fig. 51: Apparent thermal denaturation curves of porcine pancreatic lipase in the presence of different concentrations of cosolvents in 0.01 M Tris-HCl buffer, pH 7.5. The absorption spectra were recorded as a function of temperature at 287 nm. (a) control in buffer (0.01 M Tris-HCl buffer, pH 7.5), (b) in 10%, (c) in 20%, (d) in 30% and (e) in 40% cosolvents. (A) in sucrose and (B) in trehalose.

3b. 3. pH stability studies of PPL in presence of selected cosolvents

The activity profile of PPL as a function of pH has shown that a sharp decrease in the activity below pH 6.0. This may be result of titration of the imidazole ring of the active site histidine (Snellman *et al.,* 2002). The pH stability study was carried out between pH 3-9. The enzyme activity was rapidly inactivated at acidic condition and about 92% loss of activity was observed at pH 4.0, where as cosolvents treated PPL was found to be more stable against pH induced inactivation.

In presence of 40% sorbitol at pH 4.0, 61% of residual activity was retained whereas in presence of 30% trehalose the residual activity was found to be 50% (Fig. 52). Compared to sorbitol and trehalose other cosolvents found to be less effective and it retained maximum of 44%, 35% and 30% residual activity at 40% xylitol, 40% sucrose and 20% glycerol respectively (Table 21). A decrease in the pH is also known to cause an increase in the hydrophobicity of proteins (Kuhn *et al.,* 1995). This suggests that a decrease in pH should be accompanied by an increase in the degree of exclusion of cosolvents from the protein domain due to the more hydrophobic nature of the protein (Kaushik and Bhat, 2003). It suggests the dramatic reduction in stability under more acidic conditions (pH < 6.0) and is explained by titration of the Ca^{2+} coordinating Asp residues (Lang *et al.,* 1996).

pH can modulate the electrostatic interactions among charged moieties of surface ionizable residues present in the proteins resulting in alterations in the conformation and stability of proteins (Ahmad *et al.,* 2001). The change in pH affects the ionization of essential residues at active site, which are involved in substrate binding and catalysis. Some ionizable residues may be located on the periphery of the active site, commonly known as nonessential residues. The ionization of these residues may cause distortion of active site cleft and hence indirectly affect the enzyme activity (Ahmad *et al.,* 2001).

The kinetic parameters of PPL was determined in presence of cosolvents at pH 4.0, where the enzyme gets inactivated as a function of substrate concentration. From the kinetic measurements the Michalies constant (K_m) values decreases in presence of cosolvents compared to control. In presence of 30% glycerol, sucrose and 40%

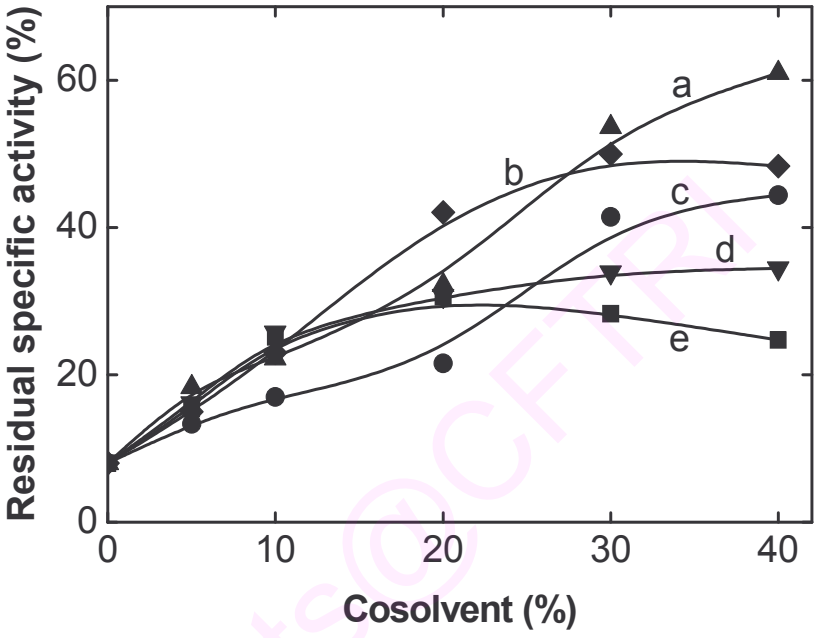

Fig. 52: pH inactivation profile of porcine pancreatic lipase in the presence of different concentration of cosolvents in 0.01 M citrate phosphate buffer, pH 4.0. (a) in sorbitol, (b) in trehalose, (c) in xylitol, (d) in sucrose and (e) in glycerol.

Table 21: Residual specific activity of porcine pancreatic lipase as a function of cosolvent concentrations at pH 4.0.

Cosolvent concentration (%) (w/v)		Residual specific activity (%)*	
		In presence of cosolvent	After removal of cosolvent
Glycerol	0**	8 ± 0.5	3 ± 0.2
	10	25 ± 2	6 ± 0.5
	20	30 ± 3	7 ± 1
	30	28 ± 3	7 ± 1
	40	24 ± 2	8 ± 1
Xylitol	0**	8 ± 0.5	3 ± 0.2
	10	17 ± 1	5 ± 0.4
	20	21 ± 2	7 ± 1
	30	41 ± 3	12 ± 1
	40	44 ± 4	13 ± 2
Sorbitol	0**	8 ± 0.5	3 ± 0.2
	10	22 ± 2	6 ± 0.5
	20	32 ± 3	9 ± 1
	30	54 ± 3	14 ± 2
	40	61 ± 4	16 ± 2
Sucrose	0**	8 ± 0.5	3 ± 0.2
	10	26 ± 2	5 ± 0.4
	20	31 ± 2	7 ± 1
	30	34 ± 2	7 ± 1
Trehalose	0**	8 ± 0.5	3 ± 0.2
	10	23 ± 2	8 ± 1
	20	42 ± 2	10 ± 1
	30	50 ± 3	14 ± 2
	40	48 ± 3	12 ± 1

* PPL in 0.01 M citrate phosphate buffer, pH 4.0.

** Controls were run parallelly in all these experiments.

xylitol, sorbitol and trehalose, K_m values were found to be 11.2, 10.4, 10.6, 7.9 and 9.3 mM respectively compared to the control value of 14.8 mM. The catalytic constant (k_{cat}) was increased in presence of above cosolvents from 5.9 to 54, 66, 85, 118 and 93×10^3 min^{-1} with increase in k_{cat}/K_m values from 399 to 4821, 6346, 8018, 14936 and 10000. This lower K_m value results in a higher catalytic efficiency with increase in catalytic constant values. These data support the idea that the affinity of the substrate towards enzyme increases in presence of cosolvents.

The changes in the secondary structure of PPL at pH 4.0 were followed in the presence of different concentration of cosolvents. The cosolvents treated enzyme shows an increase in rotations from 205 to 240 nm. From the Figure 53A & B, it is clear that the interaction of cosolvents with PPL resulted in a very small change in the CD spectra of PPL by increasing the α-helical content from 15% to 21%, 20%, 19%, 18% and 22%, in the absence and presence of 20% glycerol, 30% xylitol, sorbitol, sucrose and trehalose concentration, with minor changes in β-structure from 45% to 33%, 28%, 29%, 27% and 28% in presence of above cosolvents with slight increase aperiodic structure.

The fluorescence emission spectra of PPL show a decrease in the fluorescence emission intensity with a blue shift (≈ 9 nm) at pH 4.0 (Fig. 54A-C & 55A-B). This blue-shift of PPL at pH 4.0 can be attributed to the conformational changes in the vicinity of the surface exposed tryptophans, leading to an internalization of hydrophobic environment.

The above results clearly suggest that all the cosolvents increased the thermal stability and pH stability of the enzyme in a concentration dependent manner. Cosolvents can stabilize the enzyme by altering the aqueous environment in numerous ways, for example, alter viscosity, density, dielectric constant, osmotic pressure, preferential hydration or interaction (Baier and McClements, 2003; Xie and Timasheff, 1997c). Since water is the environment in which proteins exist and operate, the structure and dynamics of the hydration water is directly linked to protein stability (Fenimore *et al.,* 2002). Hence the concept of the effects of stabilizing solutes on the conformational changes on the enzyme functions is critical. Due to preferential

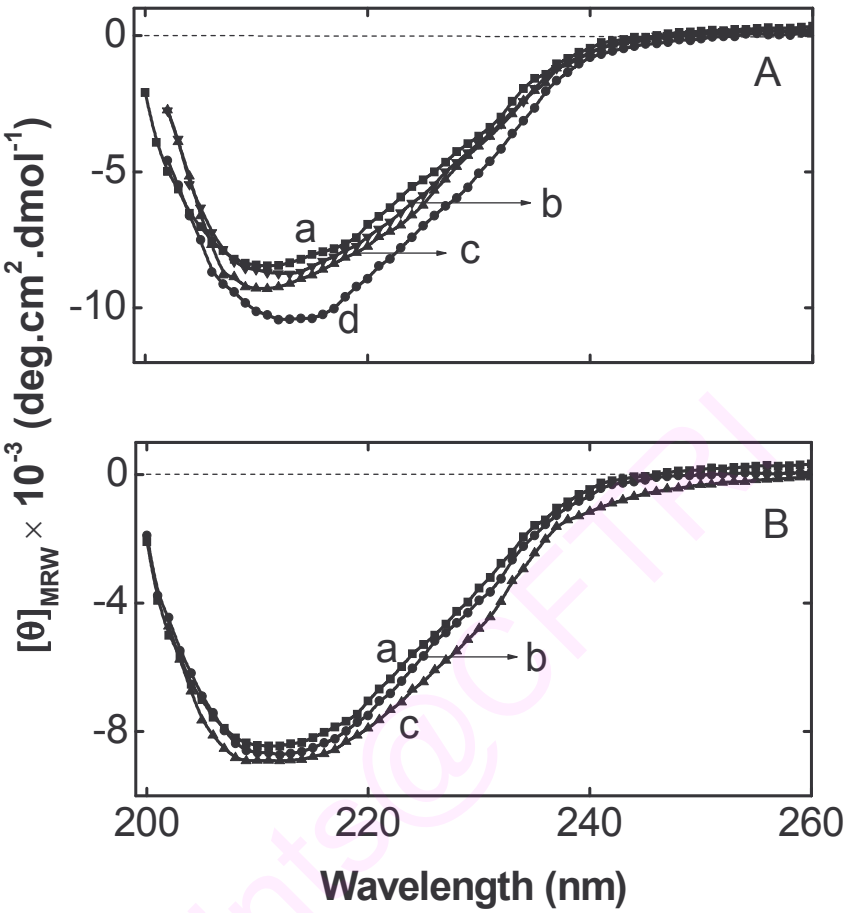

Fig. 53: (A) Far UV-CD spectra of porcine pancreatic lipase in the presence of different concentrations of cosolvents in 0.01 M citrate phosphate buffer, pH 4.0 at 25°C. (a) control in buffer (0.01 M citrate phosphate buffer, pH 4.0), (b) in 30% sorbitol, (c) in 30% xylitol and (d) in 20% glycerol.

(B) Far UV-CD spectra of porcine pancreatic lipase in the presence of different concentrations of cosolvents in 0.01 M citrate phosphate buffer, pH 4.0 at 25°C. (a) control in buffer (0.01 M citrate phosphate buffer, pH 4.0), (b) in 30% sucrose and (c) in 30% trehalose.

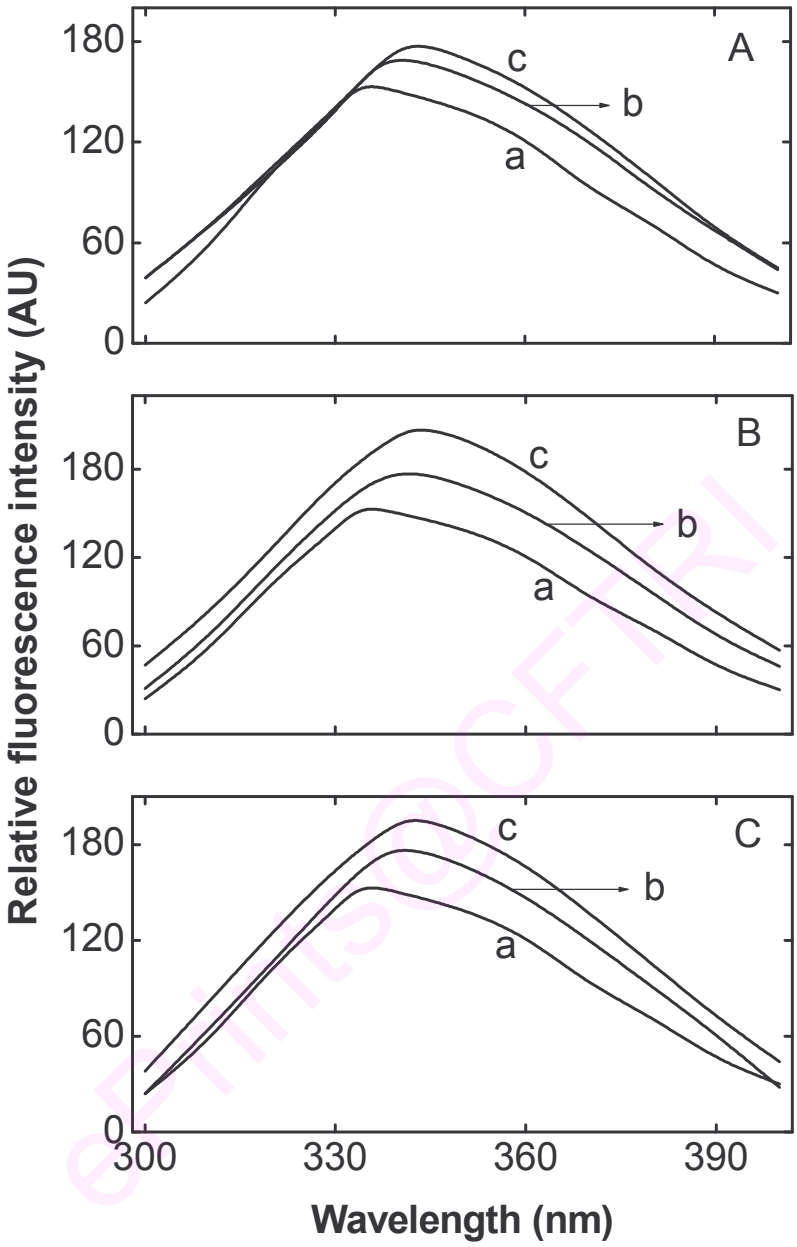

Fig. 54: Intrinsic fluorescence emission spectra of porcine pancreatic lipase in the presence of different concentrations of cosolvents in 0.01 M citrate phosphate buffer, pH 4.0. Emission spectra recorded over a range of 300-400 nm. (a) control in buffer (0.01 M citrate phosphate buffer, pH 4.0), (b) in 20% and (c) in 30% cosolvents. (A) in glycerol, (B) in xylitol and (C) in sorbitol.

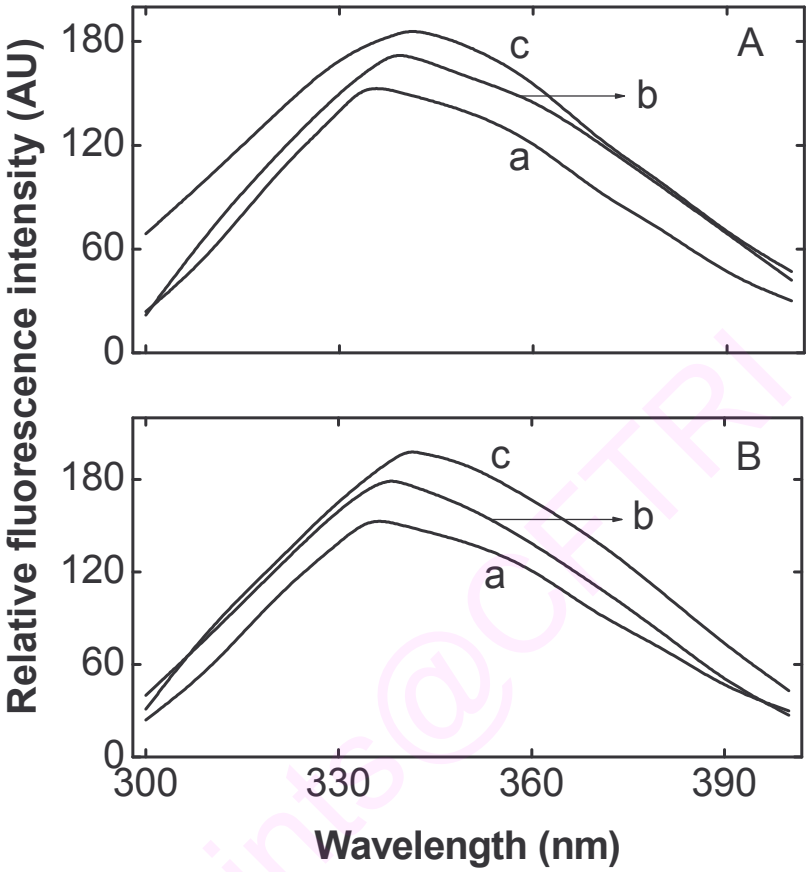

Fig. 55: Intrinsic fluorescence emission spectra of porcine pancreatic lipase in the presence of different concentrations of cosolvents in 0.01 M citrate phosphate buffer, pH 4.0. Emission spectra recorded over a range of 300-400 nm. (a) control in buffer (0.01 M citrate phosphate buffer, pH 4.0), (b) in 20% and (c) in 30% cosolvents. (A) in sucrose and (B) in trehalose.

exclusion, the presence of a stabilizing solute will tend to compress the native state ensemble of an enzyme to a more compact and narrowly distributed range of microstates leading to lower in K_m values with increase in catalytic constant k_{cat} in presence of cosolvents (Fields *et al.,* 2001).

The partial specific volume measurement shows that preferential interaction parameter dominates the protein stability of the enzyme. The effects of cosolvents on protein stability can be explained by its preferential exclusion from the protein surface. Protein surface area increases in denaturing conditions. Any state of the protein that has an increased surface area should be thermodynamically less favorable than more compact states. A critical assumption for this conclusion is that the degree of preferential exclusion varies directly with protein surface area and is not altered by changes in the chemical properties of the exposed surface. Furthermore, it is primarily the unfavorable interactions of the peptide backbone with cosolvents, which give rise to the increased protein chemical potential in solutions (Kaushik and Bhat, 2003; Kendrick *et al.,* 1997; Lee and Timasheff, 1981).

The increment in the apparent denaturation temperature of the PPL in presence of cosolvents was concentration dependent. The structuring of water in presence of cosolvents appears to be dominant factor that governs PPL stability. It is proposed that enhancement of surface hydrophilicity leads to an increase in the essential energy for exposing hydrophobic groups to water in the unfolding process. In other words, tendency to protect the native structure increases since there are non polar residues in the vicinity of the protein, cosolvents are known to increase the thermal stability by a combination of interaction such free energy changes, solvophobic, and surface tension effect with proteins (Arakawa and Timasheff, 1982a; Bolen and Baskakov, 2001; Kaushik and Bhat, 1998; Tiwari and Bhat, 2006; Xie and Timasheff, 1997c).

Cosolvents are known to alter the structure of water in the vicinity of protein molecules (Back *et al.,* 1979; Timasheff, 1993). It is therefore anticipated that any quenching effects that water may exhibit on electronically excited states of aromatic side chains, will change upon addition of cosolvents. In a defined system one can use fluorescence, especially in the presence of cosolvents only to get an indication of the changing tryptophan microenvironment and must be interpreted with great caution.

Therefore, the blue shift observed in the λ_{max} in presence of cosolvents at higher temperature indicated the increased apolar microenvironment around tryptophan.

Far UV spectrum of the protein arises primarily from the spatial arrangements of amide groups (Sreerama and Woody, 1993), ability of cosolvents to stabilize proteins against denaturing stresses originates from the unfavorable interaction of the osmolyte with the peptide backbone (Bolen and Baskakov, 2001). Because the peptide backbone is highly exposed to cosolvents in the denatured state, the osmophobic effect preferentially raises the free energy of the denatured state, shifting the equilibrium in favor of the native state.

It is clear from the above data that by the various parameters of activity, stability, kinetic and structural measurements of porcine pancreatic lipase at elevated temperature and lower pH values in presence of different cosolvents clearly suggest that all cosolvents used in this study such as glycerol, xylitol, sorbitol, sucrose and trehalose increased the thermal and pH stability in a concentration dependent manner. These data clearly suggest that, though all the cosolvents used in this study tend to stabilize protein, the mechanism by which individual cosolvent bring about the stability may be different depending upon the nature and concentration of the cosolvent used. From the studies, it is clear that there is a considerable increase in the thermal and pH stability of porcine pancreatic lipase in presence of these cosolvents as a result of preferential hydration and cosolvents stabilize proteins by shifting the denaturation equilibrium toward the native state at lower pH values. Thus the restructuring of water in presence of cosolvents appears to be the dominant factor that governs such a stabilization process of the enzyme.

SUMMARY AND CONCLUSIONS

SUMMARY AND CONCLUSIONS

The interrelationships between protein stability, function, and internal dynamics play extremely important roles in protein design. Many and different structural principles have been postulated for the increased stability in presence of cosolvents. To improve the stability of enzymes, one must understand the thermodynamic, kinetic, and structural impact of cosolvent on enzyme activities. To understand the mechanism of cosolvents mediated stability of proteins in detail, effect of cosolvents on the stability of three well characterized enzymes (invertase, nuclease P1 and lipase) were studied in presence of different concentration of cosolvents at varying temperature and pH values and interaction studies with rice bran lipase using selenium, lithium and azadirachtin.

Invertase is one the most widely used industrial enzymes that catalyzes the hydrolysis of sucrose into glucose and fructose by recognizing the fructose moiety of sucrose. Invertase from *Candida utilis* is a glycoprotein composed of two identical subunits with a molecular mass of 150 kDa each. Invertases belong to family 32 of glycosyl-hydrolase based on the beta-structure. They share a common defining feature in two critically located acidic residues, which make up the catalytic machinery responsible for the cleavage of glycosidic bonds.

Nuclease P1 from *Penicillium citrinum* is a 36 kDa glycoprotein, catalyzes the phosphodiesters bond specifically in single stranded DNA/RNA. It consists of 270 amino acid residues and contains two disulfide bonds. The secondary structure consists mainly of helices that are arranged around the 31-residue-long C-terminal α-helix. Denaturants such as GuHCl and urea which have been used for protein denaturation studies but their exact mechanism of action is still not understood the effect of low concentration of these denaturants on the stability of nuclease P1 were also studied.

Lipases are ubiquitous enzymes which catalyze the breakdown of fats and oils with subsequent release of free fatty acids, diacylglycerols, monoglycerols and glycerol. In our study lipases from two different sources such as rice bran lipase and

porcine pancreatic lipase were selected. Rice bran lipase contains 274 amino acids with a molecular weight of 30 kDa. Porcine pancreatic lipase is a glycoprotein composed of 450 amino acids with a calculated molecular weight of 50 kDa. The enzyme is an N-glycosylated consists of six disulfide bridges and two free cysteines. The 3D structure revealed that the enzyme is a two domain protein. The larger N-terminal domain consists of α/β structure containing the catalytic triad and the smaller C-terminal domain assumes a β-sandwich. The mechanism of action and substrate binding of theses two enzymes were reported to be similar.

In order to understand the properties better the structure and function of the enzymes is investigated in presence of cosolvents. Thus the detailed elucidation of the mechanism responsible for stabilization and destabilization of these enzymes is the focus of this investigation. The fundamental observation of the stabilization of macromolecules in a three-component system has a great potential for stabilization of enzymes and proteins. The properties of proteins in cosolvents underline the very fundamental concept of preferential exclusion and/or preferential interaction of such cosolvents with these macromolecules. The thermal stability, pH stability and conformation of globular proteins depend on the concentration and nature of cosolvents present in the surrounding aqueous phase.

Comprising the above aspects, the title of the thesis **"Effect of cosolvents on the structure, function and stability of selected enzymes"** is formulated.

The investigation is divided into following chapters:

Chapter 1. Studies on invertase

1. *Effect of cosolvents on the activity, stability, structure-function, kinetics and preferential interaction studies of invertase.*

Chapter 2. Studies on nuclease P1

2a. *Effect of cosolvents on the activity, stability, structure-function and kinetic studies of nuclease P1.*

2b. *Effect of denaturants on the activity, stability, structure-function and kinetics of nuclease P1.*

Chapter 3. Studies on lipases

3a. Effect of selected ligands such as metal ions and azadirachtin on the inhibitory effect on rice bran lipase.

3b. Effect of cosolvents on the activity, stability, structure-function, kinetics and preferential interaction studies of porcine pancreatic lipase.

The results obtained from above investigation are presented in the form of a thesis. Brief outlines and highlights of the results obtained in the present investigation are summarized below.

Chapter 1 describes the effect of selected cosolvents such as glycerol, xylitol and sorbitol on the activity, stability, structure-function, kinetic and preferential interaction parameters of invertase. The influence of these cosolvents at various concentrations at different pH and temperature condition were investigated thoroughly and presented in this chapter.

The above cosolvent enhances the enzymatic activity. The activity is enhanced by about 1.4 fold in 30% glycerol, 1.6 fold in 25% xylitol and 1.2 fold in 15% sorbitol respectively. This finding is supported by measurement of k_{cat} values in presence of cosolvents. The k_{cat} value in 30% glycerol, 25% xylitol and 15% sorbitol were found to be 128, 145, 113×10^3 min^{-1} respectively compared to a control value of 92×10^3 min^{-1}. There was no significant change in the structural features of invertase in presence of cosolvents at optimum condition as evidenced from fluorescence and far UV-CD spectra. Thermal stability studie shows that native enzyme loses nearly 90% of its activity at 80°C in the absence of cosolvents. In presence of cosolvents the enzyme retained maximum of 80% of its activity in presence of 40% xylitol. In case of 40% glycerol the enzyme retained 70% of its activity. The kinetic analysis of invertase at higher temperature (80°C) suggests that there is decrease in apparent K_m values from 34 mM in the absence of cosolvents to 18, 17 and 21 mM in presence of 40% glycerol, xylitol and sorbitol with increase in k_{cat} values from 9 to 65, 74 and 41×10^3 min^{-1} in the presence of 40% glycerol, xylitol and sorbitol respectively. This shows that that enzyme is more efficient and stable in presence of above cosolvents. A secondary structural study shows minor increase in both α-helical and beta sheet content of the enzyme. Fluorescence spectra show blue shift in the emission

maximum as a function of cosolvent concentration. An apparent thermal denaturation study shows that maximum increment in apparent T_m of 85°C in presence of 30% glycerol as compared to the control value of 75°C.

pH stability studies shows that cosolvents were able to protect the enzyme activity against pH changes. An increase in pH beyond optimum caused a rapid inactivation of the enzyme and 94% loss in activity was observed at pH 9.0. Glycerol did not show any protecting effect. Only xylitol and sorbitol shows protection against pH denaturation. In case of xylitol, 68% of the activity is retained at 40% concentration and sorbitol show 48% retention at 40% respectively. Kinetic parameters shown variable changes in presence of cosolvents at alkaline pH. At alkaline pH the secondary structural content of invertase were not affected significantly in presence of cosolvents. There was a decrease in emission intensity with red shift in presence of higher pH, but in presence of cosolvents there was a moderate increase in emission intensity with blue shift in the emission maximum. The preferential interaction parameter of enzyme in presence of cosolvents were determined and found to be maximum (−0.134 g/g) in presence of 40% glycerol and minimum (−0.027 g/g) in 10% sorbitol respectively. Preferential hydration parameter was found to highest (0.298 g/g) in presence of 20% glycerol and lowest (0.161 g/g) in 30% sorbitol. From the above results it is evident that the cosolvents are preferentially excluded from the immediate vicinity of protein and the positive hydration parameter shows that protein is preferentially hydrated which leads to increase in the protein stability.

Chapter 2a addresses the specific interaction of cosolvents such as glycerol, sucrose and trehalose, and their effect on structure-function, stability and kinetic studies of nuclease P1. The effect of these cosolvents at various concentrations at different pH and temperature condition were investigated and presented in this chapter.

Activity studies at optimum condition showed increase in activity in presence of cosolvents, glycerol found to be the best enhancer of activity up to 2.1 fold at 20% concentration followed by sucrose and trehalose with an enhancement of 2 and 1.5

fold at 5% sucrose and 20% trehalose concentration respectively. This indicating that, activity enhancement depends on individual cosolvents. The kinetic studies showed an increase in apparent k_{cat} value from 12×10^3 min^{-1} to a maximum of 27×10^3 min^{-1} in presence of 20% of glycerol with decreasing value of K_m which leads to increased catalytic efficiency of enzyme. The structural studies show that molar ellipticity of nuclease P1 increased in the presence of cosolvents. The intrinsic fluorescence spectra of enzyme in presence of cosolvents did not show much effect on both fluorescence emission intensity and emission maximum indicating less or no disturbance of protein surface properties.

From the thermal stability studies, in presence of all the above cosolvents results indicating that it was protecting the enzyme activity from thermal denaturation by retaining the residual specific activity to a maximum of 89% at 30% trehalose concentration. Glycerol and sucrose found to be less effective compared to trehalose with retention of 79% and 41% at 25% glycerol and 40% sucrose concentration respectively. Kinetic studies at higher temperature (80°C) in presence of cosolvents followed the similar pattern with increase in catalytic constant with decrease in K_m values. In presence of different concentrations of cosolvents both shift in band position and increase in ellipticity values reflects the stabilizing effect of cosolvents with increase in both α-helical and β-sheet content in the protein. The thermal stability of the enzyme was found to be significantly increased in presence of cosolvents as been shown by increment in the apparent thermal denaturation temperature. The maximum increment of 14°C was observed in presence of 30% trehalose.

At alkaline condition nuclease P1 loses 95% activity, upon incubating with different cosolvents, trehalose found to be the maximum stabilizer of activity followed by glycerol and sucrose. Trehalose retained up to 96% activity in 30% concentration, followed by glycerol and sucrose with a maximum protection of 67% and 59% in 30% glycerol and 10% sucrose respectively. The K_m value of nuclease P1 in alkaline condition in presence of 30% trehalose was found to be 1.35 mg compared to control value of 3.3 mg, with increase in k_{cat} values from 0.6 to 12×10^3 min^{-1}. These finding show that enzyme is more efficient in catalyzing the reaction in presence of trehalose. In presence of 20% glycerol, sucrose and 25% trehalose the α-helical

content of the enzyme was 36, 37 and 37% respectively compared to a control value of 28%. The presence of cosolvents resulted in the blue shift in emission maxima of 6 nm with increase in fluorescence intensity.

Chapter 2b describes the effect of denaturants such as urea and/or GuHCl at low concentrations on the activity profile of nuclease P1. The presence of low concentrations of denaturants resulted in significant enhancement in the activity of the enzyme. The maximum of 3.9 and 3 folds of enhancement was observed in presence of 1 M urea and 0.05 M GuHCl respectively. Kinetic studies showed a decrease in K_m from the control value of 1.1 mg to 0.86 mg and 0.6 mg in the above concentrations with an increase in k_{cat} values. At these concentrations of above denaturants it did not show any significant changes in the secondary structural content of the enzyme as evidenced by far UV-CD spectra. The apparent denaturation temperature shifted to 77 and 80°C from control value of 75°C in presence of 0.5 M urea and 0.1 M GuHCl respectively. These results indicate that increase in the thermal stability of nuclease P1 at lower concentrations of denaturants.

Chapter 3a deals with the effect of selected ligands such as selenium, lithium and azadirachtin on the structure-function and activity of rice bran lipase (RBL). Both the ligands showed inhibitory effect on the RBL. Lipase loses nearly 78% of its activity at 1×10^{-3} M concentration of selenium dioxide and was totally inactivated above 1×10^{-2} M. In the case of lithium sulfate 72% of the enzyme activity was lost at 1×10^{-3} M. Kinetic studies showed an increase in K_m values, indicating that the affinity of substrate for the enzyme decreased with increasing concentration of selenium and lithium confirming competitive type of inhibition. The far UV-CD spectra showed no significant change in the secondary structure of RBL in presence of above ions. Fluorescence studies were performed to probe the role of these ions on lipase structure. In presence of above ions 20% decrease in the emission intensity followed by 2 nm red shift in the emission maximum which attributed to the perturbation of the microenvironment of the tryptophan residues.

The fresh rice bran is prone to lipase activity. The presence of azadirachtin inhibited the lipase activity as it is evidenced by decrease in free fatty acid content of

rice bran from 16% to 3.6% after 120 hours. The kinetic studies showed that the azadriachtin inhibited the lipase activity competitively. This is indicated by increase in the K_m from 3.4 to 33 in presence of 1×10^{-3} M of azadirachtin. Interaction of azadirachtin with lipase results in decrease in α-helical content of the enzyme studied from far UV-CD spectra. Fluorescence emission intensity of azadirachtin treated rice bran lipase decreases 60%, with a 7 nm red shift in the emission maximum. The above result explains the structure-function relationship of rice bran lipase with an ultimate goal of exploiting the potential source for producing stabilized rice bran.

Chapter 3b describes the effect of selected cosolvents such as glycerol, xylitol, sorbitol, sucrose and trehalose on the activity, stability, kinetic, structural and preferential interaction studies of lipase from porcine pancreas. The study is carried out under different concentrations of cosolvents and its effect on lipase from porcine pancreas under different conditions were detailed and discussed. At optimum condition the above cosolvents did not show any effect on the enzymatic activity and stability.

Thermal stability study was performed both in presence and absence of above cosolvents. Trehalose found to be the best stabilizer of activity at higher temperature with retention of 78% activity at 40% trehalose concentration. The stability at higher temperature depends on the individual cosolvents with a maximum retention of 66, 60, 53 and 21% of activity at 40% sorbitol, xylitol, sucrose and 30% glycerol concentration respectively. Kinetic parameters showed decrease in K_m values from 19 mM in the absence of cosolvents to 12, 9, 8, 11 and 7 mM in the presence of 30% glycerol, 40% xylitol, sorbitol, sucrose and trehalose concentration, with an increase in k_{cat} values from 3×10^3 min^{-1} to 41, 116, 128, 101 and 152×10^3 min^{-1}. The decrease in the K_m value in presence of cosolvents indicates the increased affinity between the enzyme and substrate with increase in catalytic constant. Far UV-CD spectra of heat treated lipase show complete loss of secondary structure; presence of trehalose retains 50% of its molar ellipticity values. A red shift of 7 nm along with 50% decrease in fluorescence emission intensity was observed at 60°C. In the presence of cosolvents there was an increase in fluorescence intensity with a blue shift. The apparent denaturation temperature of lipase was found to be 48°C which increased in presence

of cosolvents in a concentration dependent manner. The maximum increase was observed in presence of 40% trehalose where the T_m shifted to 66°C.

The pH stability studies show that cosolvents were able to protect the enzyme activity against pH changes. At pH 4.0 lipase, loses more than 92% of its activity, in presence of 40% sorbitol it retained the activity up to 61%. The presence of cosolvents increased the catalytic constant with decrease in K_m values. These kinetic results indicated the protection effect of cosolvents against pH induced inactivation of lipase. The far UV-CD studies at acidic condition revealed minor changes in the secondary structural content, whereas in presence of cosolvents at acidic condition lipase was able to retain all of its secondary structural content. All the above findings were further supported by measurements of preferential interaction parameters of lipase in presence of different concentration of cosolvents. The preferential interaction parameter was maximum in 30% glycerol with a value of −0.083 g/g and the lowest of −0.019 g/g in 10% xylitol. The preferential interaction parameter of lipase in presence of sorbitol and sucrose found to be linearly increased with increase in concentration. The negative preferential interaction parameter of lipase in presence of cosolvents indicate that enzyme is preferentially hydrated which leads to stabilization of enzyme.

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